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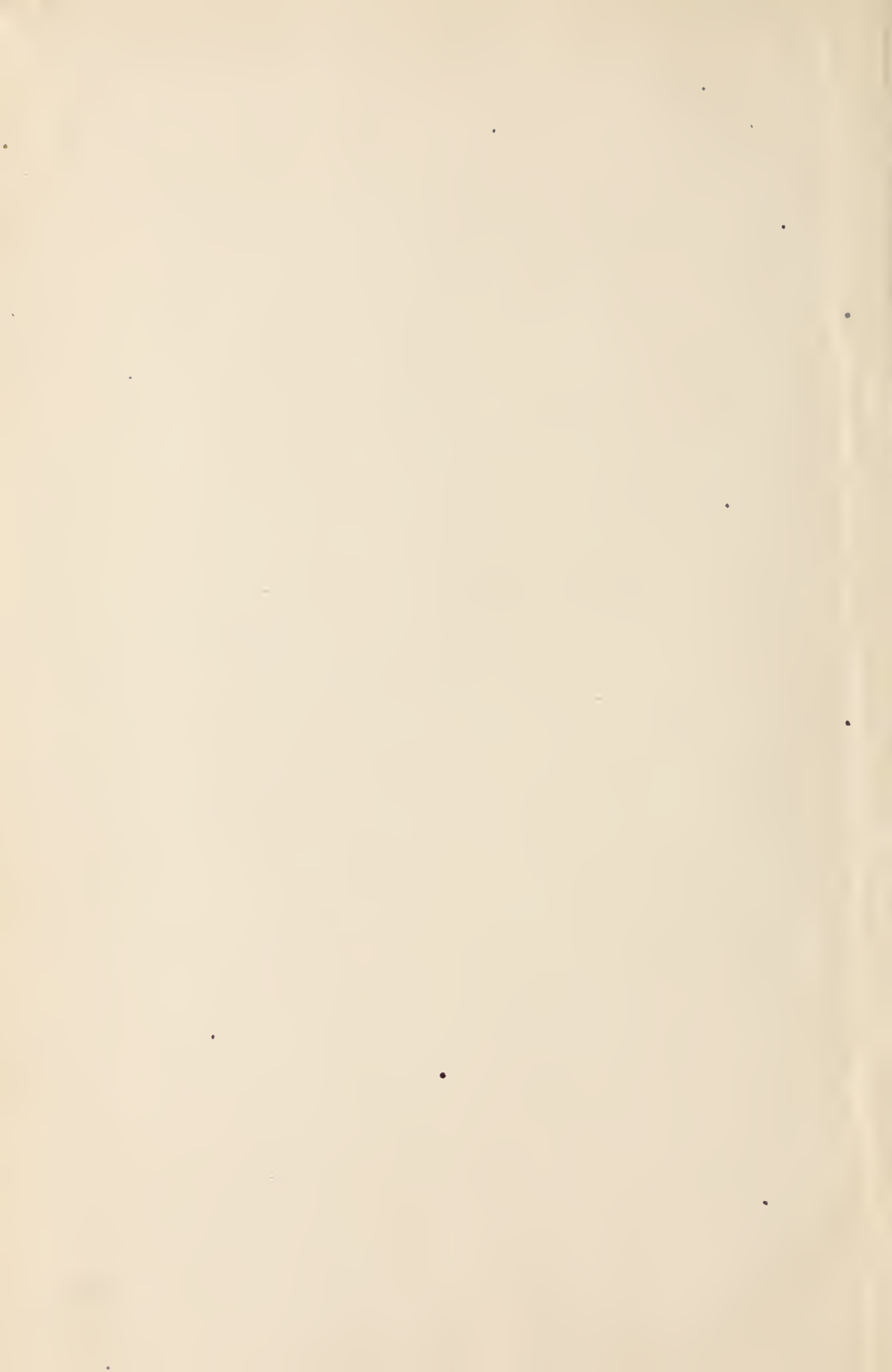
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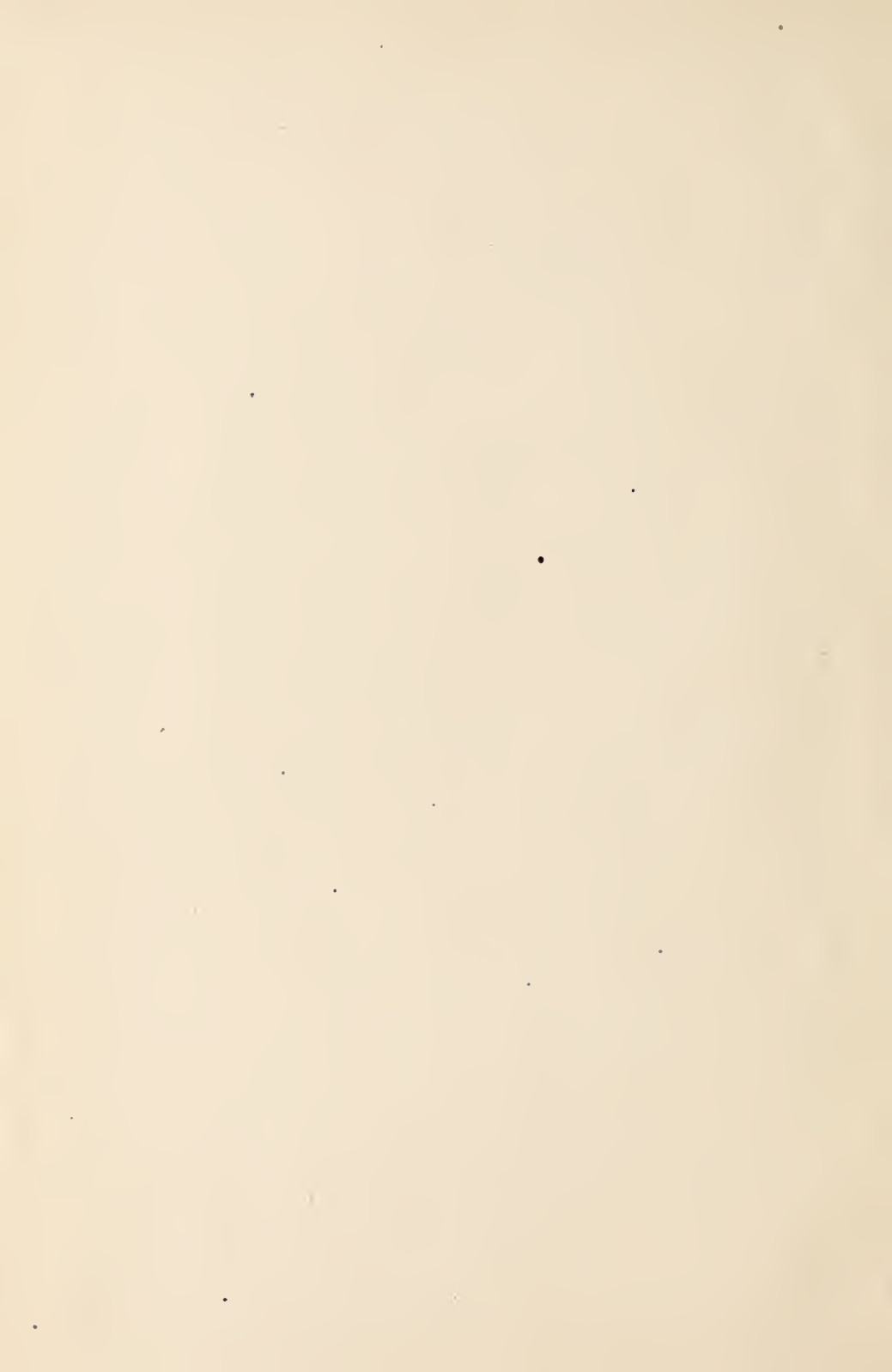
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THE
JOURNAL OF INFECTIOUS DISEASES

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Article by Hektoen, The Influence of the X-ray on the Production of Antibodies, Vol. 17, No. 2, p. 419, line 22, should read "The results of this experiment in which the exposures were practiced—"



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The
Journal of Infectious Diseases

Published by the Memorial Institute for Infectious Diseases

EDITED BY
LUDVIG HEKTOEN AND EDWIN O. JORDAN

IN CONJUNCTION WITH
FRANK BILLINGS F. G. NOVY
W. T. SEDGWICK H. GIDEON WELLS

Volume 17
1915

Chicago, 1915

Composed and Printed by
American Medical Association Press
Chicago, Illinois U. S. A.

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The Journal of Infectious Diseases

PUBLISHED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 17

July, 1915

No. 1

THE ETIOLOGY OF TYPHUS EXANTHEMATICUS*

(WITH PLATE 1)

HARRY PLOTZ, PETER K. OLITSKY, AND GEORGE BAEHR

(From the Pathological Laboratory, Mount Sinai Hospital, New York)

I. BACTERIOLOGIC STUDIES

HARRY PLOTZ

In a preliminary communication¹⁰⁵ in 1914, I described an organism which I considered to be the etiologic agent in typhus exanthematicus. The organism, a bacillus, was recovered from the blood of five cases of European epidemic typhus fever and of six cases of the mild endemic form of the disease, known in the United States as Brill's disease and designated in these papers endemic typhus fever. Since that time, I have had the opportunity of studying a much larger series of cases with the same cultural methods, and the results, together with the serologic and experimental studies, have confirmed the opinion then expressed. At the present time, we shall give only those data which bear upon the question of etiology, leaving other phases of the work for future reports.

A variety of organisms, bacilli, cocci, spirilla, and protozoa, have been reported as being recovered from cases of epidemic typhus

* Received for publication April 14, 1915.

For the financial support of these investigations, the authors are indebted to two contributors who desire to remain anonymous.

fever. Some were only asserted to have been seen in stained specimens of the blood, others were reported to have been cultivated from the blood with more or less regularity. I shall not describe these findings because none of them corresponds to the organism which I have isolated. In particular, as will be seen later, I was unable to confirm the work of Rabinowitsch,^{109, 110, 111, 112, 113, 114, 115} whose work seemed more plausible than that of his predecessors.

The endemic form of typhus fever is characterized by its comparatively mild course and very low mortality. We owe its differentiation from other fevers seen in New York, especially short term typhoids, to the clinical insight of Dr. Nathan E. Brill.²⁵ Some years after the attention of the profession had been called to this condition, its similarity to typhus fever was noted. A Committee appointed by the Section of Medicine of the New York Academy of Medicine¹¹⁶ stated in their report to the Academy on December 20, 1910, that they were convinced that the cases described by Brill were not instances of typhoid or paratyphoid fever. They were unwilling to decide whether these cases represented an atypical form of some already recognized disease, or were instances of a disease previously unrecognized. Shortly after this, Louria⁷⁶ and G. A. Friedman⁴⁵ published papers emphasizing the resemblance of these cases to cases of mild typhus fever which they had seen in Europe.

The striking cross immunity experiments of Anderson and Goldberger⁶ demonstrated that the disease described by Brill was most likely a mild form of typhus fever. They showed that the blood from cases of this disease was infective for monkeys, these animals developing, after a varying incubation period, a febrile reaction which ended by crisis, or rapid lysis, their blood being infectious for other monkeys and guinea-pigs. They also proved that animals which had reacted to the inoculation of such blood were immune to inoculation with the blood from cases of Mexican typhus fever. Likewise, animals that had reacted to inoculation of blood from cases of Mexican typhus fever were refractory to the inoculation of blood from cases of the type described by Brill.

I began my studies with the endemic form of the disease, using the viewpoints that had been established regarding typhus fever. It was very fortunate that after I had isolated an organism in a number of cases, the unusual opportunity was afforded me of investigating

cases of classical typhus fever, and of definitely establishing the identity of the two conditions.

In the search for the etiology of typhus fever, one of the most important aids was the fact that the virus of typhus fever had been demonstrated as existing in the circulating blood during the febrile period of the disease. This was first proved by the human experiment of Moczutkowski,⁸¹ who inoculated himself with typhus blood and, after an incubation period of eighteen days, developed the typical symptoms of the disease. This result was confirmed by the human experiments of Otero,¹⁰² and of Yersin and Vassal,¹³⁵ and the animal experiments of all subsequent workers, Nicolle,⁸⁸ Anderson and Goldberger,¹⁶ Ricketts and Wilder,¹¹⁷ Gavino and Girard,⁵¹ and McCampbell.⁷⁹

The possibility that the virus might be ultramicroscopic—a possibility improperly stated as a fact in some text books—was suggested by a single dubious experiment of Nicolle,⁹⁸ which neither he himself⁹² nor Ricketts and Wilder,¹³¹ Anderson and Goldberger,¹⁶ nor Gavino and Girard⁵¹ was subsequently able to confirm. In this experiment, a monkey which had been inoculated with Berkefeld filtered serum (from clotted typhus blood) presented an elevation of temperature of 0.5 C. between the sixteenth and eighteenth days after inoculation. This animal when subsequently inoculated with typhus blood did not react. The criticism of Anderson and Goldberger¹⁶ that the frequent occurrence of a natural immunity in monkeys must be considered as a possible explanation of this refractoriness on the part of the particular monkey to re-inoculation of the virus, completely upsets the value of this single experiment. On the contrary, the subsequent work of Nicolle,⁹² Ricketts and Wilder,¹¹⁷ Gavino and Girard,⁵¹ and especially of Anderson and Goldberger,¹⁶ all points to the fact that the virus of typhus fever is non-filtrable. We may assume then that the virus is of microscopic size.

The fact that the virus of typhus fever is not filtrable was probably the stimulus which induced Ricketts and Wilder¹¹⁹ to examine the blood microscopically in a number of cases. These investigators described a bacillus which they saw in blood smears stained with the Giemsa method. This bacillus apparently does bear a certain morphologic resemblance to the organism which I have cultivated, but one can not prove the identity of these two organisms by morphologic criteria alone. As the reaction of this organism to Gram's stain

is not mentioned, we are thrown further in doubt because a number of morphologically similar organisms, gram-negative and gram-positive, have been described in typhus fever. There is no evidence, then, that the bacillus seen by Ricketts and Wilder is the same as that which forms the basis of this report. Fried and Sophian,⁴⁴ altho they made a prolonged microscopic search, could find no organisms in smears of blood made from five cases of endemic typhus fever. It is of interest that these observers described in the blood of a variety of cases of bacteremia the frequent occurrence of bodies which morphologically correspond to bacilli and which are decolorized by Gram's stain.

It was surmised that, since typhus fever had been proved to be an insect-borne disease, a protozoan origin would needs be found; but Ricketts and Wilder,¹¹⁹ in refutation, called attention to the fact that the flea carries the bacillus of plague, as does the tick the spirillum of African tick fever. The same authors also pointed out that typhus fever is an acute, self-limited disease and that one attack confers immunity—features characteristic of many bacterial diseases but extremely unusual in protozoan infections.

The evidence here adduced indicates therefore, first, that the virus of typhus fever is present in the blood during the febrile period of the disease; second, that it is non-filtrable, and therefore most likely of microscopic size; and third, that it is probably of bacterial, rather than of protozoan nature. These facts actuated the majority of recent workers, including myself, to attempt to cultivate the virus from the blood of cases of typhus fever. For this purpose aerobic methods of culture had generally been used, notably by Anderson and Goldberger¹⁶ and Ricketts and Wilder,¹¹⁹ with negative results.

In the Laboratory of the Mount Sinai Hospital, the blood of over three hundred cases of endemic typhus fever has been studied by aerobic methods, with uniformly negative results. The media used were plates of agar and glucose agar (often with ascitic fluid added) and flasks of broth and 2 percent glucose broth (both with and without ascitic fluid), the flasks containing 100 c.c. and occasionally 250 c.c. of fluid.

In the search for the cause of typhus fever, I not only repeated the aerobic methods of other workers, including Rabinowitsch,^{109, 115} Pretjetchensky,^{106, 107} Mueller,^{84, 85} and Fuerth,^{47, 48, 49} with negative results, but also used anaerobic methods of cultivation—a procedure which curiously enough had not been previously employed.

Up to the present time, eleven cases of European epidemic typhus fever and forty cases of the local endemic form of the disease have been studied. Most of the epidemic cases occurred in individuals returning to America after the Balkan wars. The diagnosis was made by Dr. Joseph J. O'Connell, Health Officer of the Port of New York, to whom I wish to express my thanks for his valuable co-operation and numerous courtesies, and also to Dr. S. S. Goldwater, Commissioner of Health of the City of New York, who kindly drew the attention of Dr. O'Connell to these investigations. Of the forty endemic cases, thirty-six were patients in the wards of the Mount Sinai Hospital, for the opportunity of studying which I wish to thank Dr. Meyer, Dr. Brill, Dr. Manges, and Dr. Libman, the attending physicians of the Hospital. For the other four cases, I am indebted to Dr. Arthur Stern, of the Alexian Brothers Hospital, Elizabeth, New Jersey, Dr. Thomas J. Dunn, of the Fordham Hospital, and Dr. E. P. Bernstein, who cultured one case at the Lebanon Hospital and turned the organism over to me for further study.

We wish to thank Dr. F. S. Mandlebaum, Director of the Laboratory of Mount Sinai Hospital, for many courtesies and for placing all the facilities of the Department at our disposal.

This work, as well as the investigations which form the basis of the subsequent papers, was carried out under the direction and with the helpful assistance of Dr. E. Libman. It was the energy and stimulus which he supplied to the work which helped us carry it to a successful conclusion.

METHODS OF CULTURE

Withdrawal of the Blood.—The antecubital space is thoroughly washed for about two minutes with tincture of green soap and warm water, followed by alcohol, ether, and a 1:1000 solution of bichlorid of mercury. A tourniquet is then applied to the arm above the elbow with sufficient pressure to produce venous stasis. A distended vein in the antecubital space is then punctured and 15 c.c. of blood withdrawn. This blood is immediately distributed among the various media.

Aerobic Methods.—The aerobic method of blood culture followed is the same as that which has been employed at the Mount Sinai Hospital as a routine procedure in over five thousand febrile cases. Two plates of agar and two of glucose agar are poured, the remainder of the blood being distributed in one or more flasks (100 c.c.) of broth and of 2 percent glucose broth. The agar, in concentration of 2-3 percent, is prepared with Liebig's meat extract, and is of 0.9-1.1 percent acidity. Before use, the tubes of agar are melted by boiling in water and are then cooled to 40 C. before the blood is added.

To each of the four tubes of plain and glucose agar, 2 c.c. of blood are added and mixed by pouring the contents back and forth into a sterile test tube. The

resultant mixture is then emptied into four Petri dishes and allowed to harden. To each of the two flasks of broth, about 2 c.c. of blood are added and the vessels gently agitated to secure thorough mixing.

Because of the report by Rabinowitsch^{100 115} that he had obtained positive blood cultures in cases of typhus fever by the use of an aerobic method, his medium was tested. It consists of equal quantities of broth and ascitic fluid with 4 percent of glycerin added. To a flask containing 100 c.c. of this medium, 5 c.c. of blood are added. This method was used in five cases of epidemic, and seven of endemic typhus fever. Into each flask, 3-10 c.c. of blood were inoculated. The results were entirely negative, altho of these cases, four of the epidemic and two of the endemic gave positive results with the anaerobic method of cultivation.

Anaerobic Methods.—In the early part of this study a number of anaerobic methods were employed without success. The first successful results were obtained by the use of the ascitic fluid, kidney tissue, liquid petrolatum medium as advocated for the cultivation of spirochætae by Dr. Noguchi, to whom I am indebted for a demonstration of his methods. By the use of this method, the organism was obtained in two cases of endemic, and one of epidemic typhus fever. The method of Liborius-Veillon⁷² proved to be more satisfactory—modified, in that serum glucose agar, originally suggested by Libman as an optimal medium, was used instead of glucose agar.

Tubes measuring 15 cm. in length and 2 cm. in diameter are employed, each containing about 20 c.c. of 2 percent glucose agar. The agar is of a 2 percent concentration, and is made up with Liebig's meat extract. The acidity of the medium varies from 0.9 to 1.1 percent.

Eight such tubes are now used for each blood culture. The medium is first melted by boiling the tubes in water, and then cooled to 40 C. before use. Into each tube, about 2 c.c. of blood are injected directly from the syringe, and to this mixture about 4 c.c. of ascitic fluid are added. The contents are then thoroughly mixed by pouring back and forth into a sterile test tube. It is essential that this procedure be carried out very gently to avoid introducing air bubbles. The tubes are then allowed to cool at room temperature, or are more rapidly cooled by immersion in a vessel containing ice water. This hastens hardening and limits the absorption of oxygen. When the medium is hardened, a layer of plain agar 2 c.c. deep is added to each tube. The tubes are then incubated at 37.5 C. and observed daily by transmitted sun light, or electric light, colonies of bacteria appearing as opaque bodies.

Serum Employed.—The success of a culture depends in great part upon the proper selection of the serum. Hydrocele fluid was used with success in the earlier cultures, but as our supply was soon exhausted, ascitic fluid was substituted. It was found that only such sera should be employed which are bile-free and have a specific gravity of 1015 or over.

The sterility of the fluid must be ascertained by aerobic and anaerobic methods. The aerobic method used consists in adding 5 c.c. of ascitic fluid to each of three tubes of a 2 percent glucose agar, each tube containing about 20 c.c. of agar. This serum agar mixture is then poured into Petri plates and incubated at 37.5 C. If growth is detected during a seven-day period of observation, the fluid is discarded.

The anaerobic method consists in adding 5 c.c. of serum to each of three tubes of 2 percent glucose agar, each tube containing about 20 c.c. of the medium. To each of another set of three tubes are added 2 c.c. of ox blood, sterilized by the formalin method described by Bernstein and Epstein.²¹ This

is done to rule out the possible presence of hemoglobinophilic organisms. These six tubes are observed for twenty days, this length of time being essential because of the occasional late development of saprophytic organisms.

It was considered important to study the bacteriology of ascitic fluids. Forty-four different specimens (for which I wish to thank Dr. Noguchi, Dr. Libman, and Dr. Bernstein) were examined with the method described. The saprophytic bacilli occurring in rabbit kidney tissue were also similarly studied. Altho a number of aerobic and anaerobic microorganisms were encountered, none having the cultural characteristics of the organism later to be described was isolated. Furthermore, antigen made from these organisms did not react in a specific manner with typhus immune serum—as shown in the paper by Dr. Olitsky.

Development of Colonies of the Organism Obtained: Subinoculations.—Colonies of the organism appear in the tubes in from three to sixteen days. They may appear at different times in the same tube. It is important to observe the tubes for a long time, twenty-one days being the arbitrary limit in my work. The tubes are opened by making a circular scratch with a file just above the site of the colony to be removed. On breaking the tube across, the agar is dropped into a sterile Petri dish. It is not necessary first to immerse the tube in carbolic acid, or bichlorid of mercury. A firm platinum loop is used to remove the agar overlying the colony. A portion of the colony is then removed and inoculated on slants of 0.5 percent glucose serum agar (one-fourth volume of serum). The inoculated tubes are placed in Buchner tubes, 20 cm. in length and 2.5 cm. in diameter, containing about 25 c.c. of a 40 percent solution of potassium hydrate and 3 gm. of pyrogallic acid. The Buchner tubes are firmly sealed with a tightly fitting rubber stopper.

After the removal of the large colonies with the loop, the remaining agar is sliced with a sterile scalpel so that any small colonies may be detected. This method of removing colonies necessitates the destruction of the medium, so that the subsequent development of colonies, which have not yet matured, is interfered with. The method of removing colonies with a capillary pipette as soon as they appear, is not as valuable because the technic is a more difficult one to acquire. Furthermore, the anaerobic value of the medium is destroyed by the introduction of the pipette.

DESCRIPTION OF THE ORGANISM

Colonies.—Colonies usually appear in the lower two or three centimeters of the tube, occasionally higher, but never within three centimeters from the top of the medium. The colony first appears as a small, opaque spot. As it grows larger, a brownish area of precipitation* develops in the medium about it. By direct sunlight or incandescent electric light (frosted bulb), the colony itself appears, round and opaque, varying from 1 to 6 mm. in diameter (including the area of precipitation), the size of the colony depending

* This phenomenon was first described by Dr. Libman^{73 74} when he advised the use of media containing carbohydrates and sera (non-coagulated) for the growth of poorly growing organisms. The phenomenon occurs if acid is produced from a given carbohydrate and if sufficient growth is present to bring the acidity up to the point necessary to cause precipitation of the protein. In fluid media, a distinct, heavy precipitate is found. In solid media, there occurs a diffuse, marked whitening of the media. When blood is also present, the area of precipitation is usually of a brownish color.

on its stage of development. On cross section, it is Y-shaped, brownish in appearance, and soft in consistency. The arms of the Y are fusiform.

Morphology.—The organism is a small, pleomorphic, gram-positive bacillus, not motile, not encapsulated, and not acid fast. Its length varies from 0.9 to 1.93 microns, its breadth being from one-fifth to three-fifths its length. Most of the organisms are straight; occasional ones are slightly curved. Coccoid forms also occur. The ends are rounded or slightly pointed. In smears from solid media, the organisms lie end to end, side by side, or at angles to one another, there being no definite arrangement. Degeneration and involution forms appear early, so that after repeated transplants the organism may assume a different morphologic appearance from the bacilli in the original culture.

The organism produces no spores. Polar bodies are occasionally demonstrable with the pyoktanin, acetic acid, Bismarck brown stain (Ljubinski⁷⁵). When present, they usually appear at one end of the bacillus, but may appear at both ends.

Cultural Characteristics.—The methods used in this study for determining the cultural characteristics of the organism are suggested as a basis for future studies of all anaerobic organisms. At present anaerobic bacteria are cultivated on such varying kinds of media that an accurate classification is not possible. This classification is based mainly on the work of Dr. Noguchi.

Besides the usual cultures grown in Buchner tubes or in Novy jars, inoculations were made in a variety of media in long test tubes, 20 cm. in length and 1.25 cm. in diameter, the type used by Noguchi for the cultivation of spirochætae.

The tubes were inoculated with saline emulsions of the organism, incubated at 37 C., and observed for twenty days. The agar, of 2 percent concentration, was made up with Liebig's extract, the acidity varying from 0.9 to 1.1 percent. The broth was similarly made up with beef extract and was of an acidity of 1.1 percent. Twenty cubic centimeters of agar or broth were used in each tube. The quality and proportion of the serum used were the same as that previously described. The tissue employed in some of the media consisted of pieces of rabbit kidney, the sterility being tested by incubation in plain broth for three days prior to use. Over some of the media a layer of about 2.5 c.c. of liquid petrolatum was used.

Table 1 shows the results obtained with the various media. A description of the results of these studies, which were made soon after the isolation of the organism, follows.

TABLE 1
RESULTS OF CULTIVATIONS IN VARIOUS MEDIA IN DEEP TUBES

	Alone	Layered with Liquid Petrolatum	Kidney Tissue	Kidney Tissue and Liquid Petrolatum	Ascitic Fluid	Ascitic Fluid and Kidney Tissue	Ascitic Fluid, Kidney Tissue, and Liquid Petrolatum
Agar	No growth	No growth	No growth	No growth	No growth	No growth	No growth
One-half per- cent glucose agar	No growth	No growth	Slight growth	Slight growth	Growth	Growth	Growth
Two percent glucose agar	No growth	No growth	Slight growth	Slight growth	Growth	Growth	Growth
Sugar-free broth	No growth	No growth	No growth	No growth	No growth	No growth	No growth
Two percent glucose broth	Very slight growth	Slight growth	Slight growth	Slight growth	Slight growth	Slight growth	Slight growth

Cultures in Deep Tubes.—The growths are described after four-day observations. Negative tubes were observed twenty days.

Agar: No growth appears in any of the media.

Glucose agar, 0.5 percent: With kidney tissue, after forty-eight to seventy-two hours, a slight growth appears about the tissue in the form of minute, white flocculi; with ascitic fluid, a more marked growth appears, extending up into the medium to within two and one-half centimeters from the top. Precipitation occurs after the growth becomes profuse. There is no odor evident in this, or any other medium.

Glucose agar, 2 percent: The same characteristics are observed as in the 0.5 percent medium, except that the growth is more profuse.

Sugar-free broth: No growth is found in any of the media. Indol tests, therefore, could not be made.

Glucose broth, 2 percent: After eight days, the growth appears as minute flocculi at the bottom of the tube with a tendency to grow up the side of the tube. The medium remains clear above the growth. No pellicle is formed.

Cultures in Buchner Tubes.—Agar: No growth appears.

Glucose agar, 0.5 and 2 percent: No growth appears.

Glucose serum agar, 0.5 and 2 percent (three parts agar, one part ascitic fluid): After three days a creamy white growth is observed with no tendency to spread over the surface but rather to heap up. The growth may occasionally take on a very light-brownish color in old cultures. There is a moderate amount of precipitation. After five days, the growth is more profuse and precipitation more marked. In subcultures, the precipitation becomes still more marked.

Potato: After four days, an invisible, whitish growth is found, demonstrable by scraping the medium.

Gelatin, 15 percent: No growth is found even in thermostat.

Litmus milk: A slight amount of acidity is observed after eighteen days.

Loeffler's medium: A very slight growth is observed.

Fermentation Reactions.—The fermentation reactions were tested by inoculation of tubes of serum agar containing various carbohydrates. It has been shown by Libman, Celler, and Sophian* that the optimal media for determining acid production are serum carbohydrate media, both fluid (broth) and solid (agar), the broth media being better for investigation of the pneumococcus-streptococcus group which they particularly studied. For the investigation of the typhus bacillus, it was decided to use the solid media because growth was found to be better on them.

The media were made up of agar of a 3 percent concentration, containing 2 percent of the various carbohydrates, to which was added one-third as much ascitic fluid (specific gravity of 1013 or over). The medium was tinged with litmus (Kahlbaum). The carbohydrates used were chemically pure glucose, lactose, saccharose, maltose, mannite, lactose (all Merck's chemicals), arabinose, inulin, raffinose, galactose, and dextrin (prepared by Kahlbaum). The studies were made with agar of which the acidity was 1 and 1.5 percent. Acid production and precipitation occurred just as often in the 1 percent medium, and so in the identification of organisms later isolated, only the 1 percent medium was employed. Surface inoculations were made, the tubes then being placed in Buchner tubes and observed after six days' incubation in the thermostat. It was found important to inoculate a fair amount of culture. All experiments were made in quadruplicate.

* Read at the meeting of the Association of American Pathologists and Bacteriologists, April, 1914; not yet published.

There were studied at first three strains of the bacillus obtained from epidemic cases, and one from an endemic case. Table 2 shows the results after six days' incubation.

These observations show clearly that the bacillus produces acid from glucose, maltose, galactose, and inulin, and does not produce acid from raffinose, mannite, arabinose, saccharose, dextrin, and lactose. Visible gas is not produced from any of these carbohydrates.

After these preliminary studies were made, a number of organisms subsequently isolated from typhus cases, or from experimental animals, were investigated in a similar fashion, and the fermentation reactions were found to be identical with those just detailed. Later in the work, organisms were identified by their reaction in all these carbohydrates, except mannite, dextrin, and arabinose. The fact that the organisms from the endemic cases differed in no way from those from the epidemic cases, is of great importance.

TABLE 2
RESULTS OF THE TESTS FOR FERMENTATION REACTIONS

	Epidemic 1		Epidemic 2		Epidemic 3		Endemic	
	A.	P.	A.	P.	A.	P.	A.	P.
Glucose	+	—	+	—	+	—	+	—
Raffinose	—	—	—	—	—	—	—	—
Maltose	+	—	+	—	+	—	+	—
Mannite	+	—	+	—	+	—	+	—
Arabinose	—	—	—	—	—	—	—	—
Galactose	+	+	+	+	+	+	+	+
Inulin	+	+	+	+	+	+	+	+
Saccharose	—	—	—	—	—	—	—	—
Dextrin	—	—	—	—	—	—	—	—
Lactose	—	—	—	—	—	—	—	—

A = acid production; P = precipitation.

Colonies on Plates.—Plates were poured from a saline suspension of the organism and grown in Novy jars at 37.5 C. The media used were agar, glucose agar, and serum glucose agar. Colonies were obtained only in serum glucose agar, appearing after seven days. Only once did surface colonies develop.

The surface colonies measured from 1.5 to 2 mm. in diameter. They were round, oval, or oblong, projecting almost 1 mm. above the surface, each colony surrounded by a zone of fine, dust-like dots, located in an area of precipitation. The color by reflected light was light cream; by transmitted light, the colonies were opaque. Microscopically (No. 6 objective and No. 4 eyepiece), the colonies appeared as opaque bodies with fading, irregular contour. The granules in the surrounding zone were irregular in form, or spherical with refractive contour, and were more numerous close to the colony.

The colonies just beneath the surface measured 1 mm. in diameter, were round, oblong, or triangular in shape, and of a light cream color. Around each colony there was a zone of precipitation, in which were localized fine, opaque granules.

The deep colonies varied in size from pin-point to 1 mm. in diameter. They were round, oval, or triangular, the last type predominating. Microscopically, the colonies were triangular, or acorn-shaped, sharply circumscribed with irregular contour.

From a study of the foregoing facts, the following deductions are made regarding the growth of the organism described: The organism grows much better in solid than in fluid media; in solid media it requires carbohydrate and serum; the optimal medium in the studies thus far made is 2 percent glucose serum agar. For keeping the organism alive, however, the 0.5 percent glucose serum agar was used, as the larger glucose content was presumed to decrease virulence to a greater extent.

Aerobic Studies.—This organism is an obligatory anaerobe. It was at first thought that slight growth had occurred aerobically, after anaerobic cultivation. This was due to the fact that a large amount of growth had been carried over in transplanting and was mistaken for further growth. Subsequent observations demonstrated that no increase in growth had occurred on the slants. Moreover, the material on the slants could not be further subcultured aerobically. Every time a culture was made from the original colony, two slants of 0.5 percent glucose serum agar were inoculated with portions of the growth. These tubes were observed for twenty days and no growth was ever noted. Subsequently, many strains of the organism were transplanted on the following media and kept under aerobic conditions, always with negative results: 0.5 percent glucose serum agar, 2 percent glucose serum agar, 2 percent glucose serum agar plus human blood, Loeffler's medium, Dorset egg medium.

Thermal Death Point.—This was found to be a temperature of 55 C., for ten minutes, a result which coincides with the results obtained by Anderson and Goldberger¹⁶ for the thermal death point of the virus in typhus blood.

Filtration Experiments.—After the isolation of the typhus bacillus, its filtrability was determined as follows: Two cubic centimeters of sterile, distilled water were added to each of several slants

of a seven-day growth of the organism. The growth was then scraped off the surface of the medium, care being taken not to remove any of the agar. This milky emulsion was again diluted with the same amount of sterile, distilled water and passed through a Berkefeld filter, size N. The filtrate was then inoculated on slants of 0.5 percent glucose serum agar, which were placed in Buchner tubes. Control tubes, made from the unfiltered emulsion of the organism, were similarly inoculated on slants of 0.5 percent glucose serum agar. In no instance did tubes inoculated from the filtrate show any growth, whereas all the controls grew in the usual manner. This agrees then with the fact that the virus in the blood is non-filtrable.

GENERAL RESULTS OF THE BLOOD CULTURES

Epidemic Cases.—Blood cultures were made in seven cases of epidemic typhus fever during the febrile stage of the disease (Tables 3 and 4). In every case studied, the bacillus was isolated in pure culture. From an eighth case, blood was inoculated intraperitoneally into two guinea-pigs, and after an incubation period they developed a typical febrile reaction. During the height of the reaction, blood obtained by cardiac puncture was found to contain the organism.

Endemic Cases.—Blood cultures were made in thirty-seven endemic cases. In thirty-four, the cultures were made at varying times during the course of the disease up to and including the day of crisis. Eighteen of these, or 53 percent, yielded the bacillus. A higher percentage of positive results would probably have been obtained, if a rich ascitic fluid had been available for use in each case.

Blood Cultures After the Crisis.—Blood cultures were made after the crisis in six of the cases of epidemic typhus fever, and all proved negative. Three of these had already been cultured during the febrile period of the disease and the organism recovered. These were Cases 2, 4, 5, 7, 8, and 9, and the post-critical cultures were made between the thirteenth and sixteenth days.

Nine of the endemic cases were cultured after the crisis. Two of them yielded the bacillus, the one twelve, and the other, thirty-six hours after the crisis. These results are of great interest in view of the fact that it has been shown by Anderson and Goldberger,¹⁶ and by ourselves (see experimental studies), that the blood of an infected animal is still infectious thirty-two hours after the crisis.

TABLE 3
CULTURAL RESULTS IN EPIDEMIC TYPHUS CASES

Number Case	Time of Culture*	Days after Culture When Positive**	Amount of Blood Cultured	Number of Colonies Found
1	36 hours before	14	6	2
2	4 days before	7, 9, 10	6	3
3	6 days before	3	Fluid medium used	
4	4½ days before	4, 5	6	14
5	3½ days before	5, 6, 8	6	28
6	At crisis	3, 5,	6	9
7	13 days after	Negative	3	0
8	16 days after	Negative	3	0
9	13 days after	Negative	3	0
10	At crisis	Contaminated
11	4 days before	7, 10, 16	12	8

* This is reckoned back from the crisis, or, if there was not a typical crisis, the time when the temperature first reached 100 F. (38 C.).

** The second and third figures refer to the later appearing colonies.

TABLE 4
CULTURAL RESULTS IN ENDEMIC TYPHUS CASES

Case Number	Time of Culture	Days after Culture When Positive	Amount of Blood Cultured	Number of Colonies Found
1	26 hours before	3	Fluid medium used	
2	12 hours before	4	Fluid medium used	
3	4½ days before	4	No record	
4	4 days before	3	No record	
5	3½ days before	6	No record	
6	36 hours after	Negative	14	0
7	36 hours after	8	8	1
8	4 days before	Negative	14	0
9	12 hours after	14	4	1
10	60 hours before	7	12	1
11	16 hours before	Negative	14	0
12	36 hours before	Negative	12	0
13	48 hours before	9	7	1
14	60 hours before	Negative	16	0
15	3½ days before	Negative	12	0
16	Day of crisis	9	15	1
17	3½ days before	Negative	20	0
18	24 hours before	9	16	1
19	Day of crisis	Negative	12	0
20	48 hours before	10, 11	14	3
21	60 hours before	Negative	12	0
22	12 hours before	Negative	14	0
23	10 hours before	11	15	1
24	No culture	—	—	—
25	36 hours before	Negative	14	0
26	4 days before	Negative	16	0
27	4 days before	Negative	16	0
28	4 days before	Negative	16	0
29	No culture	—	—	—
30	3 days before	Negative	15	0
31	Day of crisis	Negative	15	0
32	8 hours before	11	15	1
33	5 days before	11	15	2
34	5 days before	9	10	1
35	No culture	—	—	—
36	Day of crisis	9, 10	16	2
37	48 hours before	Negative	12	2
38	24 hours before	7, 8	10	2
39	24 hours before	8	14	4
40	5 days before	10	15	9

RESULTS OF ANAEROBIC CULTURES IN CONTROL CASES

With the identical technic used for isolating the bacillus just described from individuals with typhus fever, anaerobic blood cultures were made in 198 febrile cases. The ascitic fluids used in these cases were the same as those employed in the typhus cultures. These cases represented a variety of conditions, such as typhoid fever, tuberculosis, acute osteomyelitis, acute rheumatic endocarditis, subacute bacterial endocarditis, chorea, acute nephritis, influenza, liver abscess, acute cholangitis, erysipelas, phlebitis, splenic anemia, post-partum infection, etc. In none of these 198 cases was an organism recovered which in any way resembled the bacillus isolated from the cases of typhus fever.

THE IDENTITY OF THE ORGANISMS ISOLATED FROM VARIOUS SOURCES

The organisms isolated from the cases of endemic typhus were found to be culturally identical with those isolated from cases of epidemic typhus. Careful studies were made in order to determine whether any small cultural differences could be detected, such as exist, for example, between the typhoid and paratyphoid bacilli. In no instance was the slightest variation observed in their behavior on any of the media.

Similarly, the organisms isolated by blood culture from eight guinea-pigs and one monkey (virus obtained from Anderson and Goldberger), in which the disease had been produced experimentally, were studied. All of them were found to possess the same cultural characteristics as those isolated from the human cases of typhus fever. The importance of the proof of the identity of the organisms isolated from epidemic, endemic, and experimental typhus fever is self-evident.

PERIOD OF MOST MARKED BACTERIEMIA

In most of the cases studied, it was impossible from the history to determine the exact date of onset of the illness. In estimating at what period of the disease the blood cultures were taken, it was therefore necessary to reckon backwards from the date of termination of the disease. The day of the crisis, or in the absence of a crisis, the day on which the temperature first reached 100 F. (38 C.) and remained at about that figure, was always considered as the last day of the disease. By adding together all the amounts of blood used for cultures made within three days before the crisis, and those made on the

fourth and fifth days before the crisis, and comparing these figures with the number of colonies found at the same times, it seems that the organism occurs more abundantly in the blood on the fourth and fifth than on the few days immediately preceding the crisis. It will be of great interest to make further studies in this connection, because in the cultures taken from the inoculated animals the organisms were found most frequently early in the disease. Therefore it may be found that blood cultures taken in the first few days of the disease in man may show the largest number of organisms.

RELATION OF THE DEGREE OF THE BACTERIEMIA TO THE SEVERITY OF THE CASES

A calculation of the total amount of blood used in all cultures made on or before the day of crisis in the epidemic and in the endemic cases gives the following result: In epidemic cases, 51 c.c. of blood were used and 74 colonies found; in endemic cases, 436 c.c. of blood were used and 33 colonies found. Therefore, in the cases which I have studied, the bacillus was found eighteen times as abundantly in the blood of the epidemic, as in the blood of the endemic cases. Without laying too much stress on this exact figure, it is very evident that the bacteriemia is more intense in the epidemic, than in the endemic cases. This difference in the number of bacteria in the blood may be one of the reasons for the marked difference in the severity of the two varieties of the disease.

The figures for the numbers of organisms found in the positive cultures and of the amount of blood used can be seen in Tables 3 and 4. It will be noted that in the two cases of epidemic typhus fever which had the most marked bacteriemia, the figures show that there were present in a cubic centimeter of blood, two and one-third, and four and two-thirds colonies, respectively. Both of these cases were fatal.

DIAGNOSTIC VALUE OF BLOOD CULTURES

Because of the late maturation of the colonies, the results of the blood cultures are usually only known after the termination of the illness. But it is nevertheless of value for confirming the clinical diagnosis. In fact, in 87.5 percent of fifty-one cases studied, the clinical diagnosis was confirmed by blood culture, agglutination, or complement-fixation tests.

On the other hand, in two cases in which the diagnosis had been overlooked, the positive blood culture first called attention to the nature of the condition. In both cases, the temperature which had been high on admission, fell suddenly to normal within twelve hours. The subsequent convalescence was uneventful and a tentative diagnosis of influenza had been made. After the results of the blood cultures were reported, a more careful investigation revealed the fact that both individuals had passed through an illness with the typical symptoms of typhus fever. The sudden drop in temperature immediately after admission undoubtedly represented the crisis.

It is quite probable, therefore, that similar cases of typhus fever, which are only recognized toward the very end of the illness, are not infrequently wrongly interpreted.

CONCLUSIONS

Attention has been drawn to the fact that the virus of typhus fever circulates in the blood of an infected person or animal, that the virus is not filtrable and, therefore, is of microscopic size, and that aerobic cultures made with a great variety of media are negative. Hence, it was considered plausible that the infective agent is an anaerobic organism. By means of an anaerobic method of blood culture, a bacillus was isolated in pure culture from seven cases of European epidemic typhus fever, or 100 percent of the cases studied during the febrile period. With the same method, the identical organism was recovered during the febrile period of the local endemic form of the disease in eighteen of thirty-four cases, or 53 percent, and in two additional cases taken after the crisis. Similar blood cultures made in 198 control cases yielded no such organism.

Considered in conjunction with the serologic and experimental evidence which follows, these facts prove that this bacillus is the etiologic factor in typhus exanthematicus.

At the very kind suggestion of Professor William H. Welch, it has been deemed advisable to name the organism, *Bacillus typhi-exanthematici*. The name *Bacillus typhi exanthematici* was applied by Klebs⁶³ in 1881 to a hypothetical organism in typhus fever. The name was never actually used, as the search for the organism proved futile. The hyphenating of "typhi" and "exanthematici" succeeds in giving us a binomial designation properly descriptive.

II. SEROLOGIC STUDIES*

PETER K. OLITSKY

The typhus bacillus, being an obligatory anaerobic organism, can not be studied with the same ease as aerobic bacteria. Hence it was necessary to modify the usual serologic methods in order to overcome certain difficulties. Indeed, the study of some reactions was restricted by the inapplicability of any method. Furthermore, at the inception of this work, two organisms were under consideration, one having been isolated from the blood of patients suffering from endemic typhus fever (Brill's disease), and the other, from the blood of individuals with epidemic (European) typhus fever. The serologic investigations demonstrated early in the course of the work that these two conditions are identical, and, what is still more important, they have definitely associated the organism with the etiology of typhus fever.

I shall discuss the various phases of this work in the following order: (1) complement fixation, (2) agglutination, (3) precipitation, (4) other antibody actions, (5) the identity of the organism obtained from endemic typhus fever with that isolated from epidemic typhus fever, (6) serologic studies in animals, (7) serologic reactions in individuals after exposure to typhus fever, (8) the relation between the serologic results and typhus fever.

COMPLEMENT FIXATION IN TYPHUS FEVER

Attempts have been made, even before a definite organism was associated with typhus fever, to study the fixation of complement in this disease. Cathoire,³³ in 1910, obtained positive reactions in typhus fever, employing as antigen alcoholic extracts of spleen tissue from a case of the disease. Likewise Markl,⁷⁸ using extracts of organs (spleen, liver, lung, and heart) from cases of typhus fever, obtained distinct inhibition, altho not complete fixation, with sera from this disease. Upon analyzing these results, one must consider the non-specificity of the results. Alcoholic extracts of tissue will yield a lipoid antigen. Such, if carefully titrated, will bind complement in the presence of "lipoidophilic" substances (as are found in the blood in syphilis). It makes little difference whether the organs are obtained from diseased or healthy subjects. Hence, there

* This work was done under the tenure of the Eugene Meyer, Jr., Fellowship.

is no relation between such results and the etiology of typhus fever. The question as to whether syphilis is present at the same time must be considered when complete fixation is found by the use of these methods. In the same category may be placed the results of Jablons,⁶⁰ who found that a lipid antigen gave positive results in several cases of typhus fever. Here again the question of syphilis being concomitantly present, must be considered.

My own work does not bear out these results. With a pure lipid (Noguchi) antigen or with alcoholic extracts of guinea-pig hearts, absolutely negative results were obtained in eleven cases of epidemic typhus fever and eight cases of endemic typhus. (Some of these tests were made by Dr. David J. Kaliski, of the Mount Sinai Hospital.) We must conclude that the results obtained with lipid antigens have no bearing upon the causative factor in typhus fever.

The results of my work are based upon the principle of specificity. Given an organism, such as the typhus bacillus, I have determined the presence of complement-fixing antibodies against this bacterium only in typhus fever, not in other conditions.

In describing the methods used, I shall state in detail only such procedures as my experience has led me to adopt.

METHOD

The Antigen.—There are several factors which should be emphasized in the preparation of the typhus antigen.

(1) Aqueous extraction is superior to saline. Autolysis, after heating, for twenty-four hours, gives a much better extract.

(2) Because of the fact that they are more easily extracted, older cultures are preferable to those more recently isolated.

(3) Antigens should have a long "range," i. e., the anticomplementary unit should be at least four times the amount used in the test. The antigenic property should be determined by titrating with immune serum.

(4) Filtrates but not suspensions of organisms are preferable. The latter are unstable, and vary in titer from time to time. Bacterial bodies per se have a tendency to absorb complement, hence the danger of non-specific reactions. Besides, suspensions are usually too anticomplementary and too little antigenic.

The method of preparing antigen is as follows: Since this organism is an obligatory anaerobe, there is no method for growing it in large bulk. The growth on a 0.5 percent glucose serum agar slant (grown 4-5 days in a Buchner tube) is washed off with 1 c.c. of sterile, distilled water. Care is taken that none of the media is transferred to the water, and each culture used is carefully examined as to its purity with a gram stain. Twenty to thirty such slants give an amount of extract sufficient for all practical needs (10-15 c.c. extract). The turbid suspension is then heated to 60 C. for one hour and then autolyzed at 37 C. for twenty-four hours. Through a sterile Berkefeld filter, size N,

which has been previously digested and cleaned out, this turbid fluid is filtered. The filtration should be slow, otherwise the extract becomes contaminated on standing, or anticomplementary as the result of the presence of bacterial bodies. No preservative is added to the resultant extract. The antigen keeps for months if, when it is first made, it is heated at 56 C. for one-half hour on each of three consecutive days. For titrating or use, it is "normalized" by the addition of nine parts of antigen to one part of 0.85 percent saline.

The "antigenic" value of the antigen is determined as follows: The antigen is first titrated for its anticomplementary unit. In this respect, the titrations of most antigens are straight (undiluted), 1:5 or 1:10. An antigen diluted 1:5 is very safe to use. One-fourth of the anticomplementary unit is titrated with decreasing amounts of rabbit immune serum, starting at 0.1 c.c. A good antigen should give complete inhibition of hemolysis with amounts of serum less than 0.005 c.c. and no inhibition with human normal serum.

The rabbit immune serum is obtained by intravenous injection of a rabbit at four-day intervals with increasing amounts of typhus culture. The initial dose amounts to the saline suspension of the growth on one slant; the last injection is equivalent to the growth on four slants. The animal is bled 9-10 days after the last injection (v. infra).

Antigen 11-9, for example, was titrated for its anticomplementary unit. There was incomplete hemolysis in 0.1 c.c. Upon dilution 1:5 complete hemolysis was obtained in 0.4 c.c., complete inhibition in 0.6 c.c. Titrating 0.1 c.c. of a 1:5 dilution with rabbit immune serum showed complete binding of complement with 0.002 c.c. of the rabbit serum. Such an antigen was regarded as trustworthy.

The Hemolytic System.—Complement is prepared by diluting fresh guinea-pig serum 1:10. This is titrated against constant amounts of amboceptor. The amount used is ordinarily 0.3 c.c. The amboceptor consists of anti-sheep cells rabbit serum. The titer is usually above 1:1000. Washed sheep cells, 5 percent suspension, employing 0.5 c.c., completes the hemolytic system. In general, the hemolytic system must be very active if clear-cut results are to be obtained. This system (which is used with success by Dr. David J. Kaliski for routine Wassermann tests) is gauged so that complete hemolysis occurs in fifteen minutes. It represents one-half the quantities recommended by Wassermann for his test.

The Patient's Serum.—Inactivated (56 C. for one-half hour) serum is used in amounts of 0.05 c.c. and 0.1 c.c. Serum that gives complete fixation in both amounts is regarded as ++++; in only the larger amount as ++ or ++++. Such positive sera hold their antibody content for over one month if kept cold.

The Test.—Each test consists of a known positive and a known negative serum, as well as the serum itself, and antigen (twice the amount used in the test) as controls. Fixation is allowed for one-half hour at 37 C., and three and one-half hours in the ice box. Readings are made when the serum and antigen controls are completely hemolyzed.

Complement Fixation During the Course of Typhus Fever.—These methods were applied in the study of typhus fever. In the beginning, three antigens were used: one made up of the epidemic

typhus organisms; one, of the organisms from endemic cases; and a third, of a mixture of both organisms. Upon comparing the results obtained in one case of endemic typhus and eleven cases of epidemic typhus fever, we find that the endemic case gave a + + + + reaction with an epidemic typhus organism, but a negative one with an endemic typhus organism. There may be grounds for this in that, at that time, we had only one strain of endemic typhus organism on hand, but four strains of epidemic typhus organisms. It is well known that polyvalent antigens almost always give stronger reactions than monovalent.

Of the eleven cases of epidemic typhus fever, nine were tested after the crisis; two of these gave negative reactions to endemic, as well as epidemic typhus organism antigen, while seven were positive with either one or the other of three antigens (Table 1). The relations of these antigens to each other we shall discuss in another place.

TABLE 1

THE REACTIONS IN ENDEMIC TYPHUS FEVER AND EPIDEMIC TYPHUS FEVER TOWARDS INDIVIDUAL ANTIGENS

Case	Days After Crisis	Individual Antigens		Mixed
		Epidemic Typhus	Endemic Typhus	
Epidemic typhus 2.....	5	Negative	++	+++
Epidemic typhus 3.....	3	++++	+++	++++
Epidemic typhus 4.....	++	++	++
Epidemic typhus 5.....	4	Negative	Negative	Negative
Epidemic typhus 6.....	9	Negative	Negative	Negative
Epidemic typhus 7.....	3	++	++	++
Epidemic typhus 8.....	5	++	++	++
Epidemic typhus 9.....	3	Negative	Negative	++
Epidemic typhus 11.....	4	Negative	Negative	Negative
Endemic typhus 8.....	6	++++	Negative	Negative

After this, we discarded these single antigens and employed one containing both strains of the typhus bacillus. Undoubtedly because of this, as well as of the fact that we acquired more strains of the organism, our results since then have been more uniform and our positive reactions more common.

Including the series mentioned, eleven cases of epidemic and thirty-four of endemic typhus fever were tested. Nine of the epidemic and thirty of the endemic cases were tested after the crisis. During this apyrexial period, the total number of positives was twenty-eight, or 71.8 percent. Of all the cases tested, irrespective of the stage of

the disease, the total number of positives was twenty-nine, or 64.4 percent. This is a rather low percentage, but one must consider that on account of the nature of the work, namely, determining whether this bacillus is the cause of typhus fever, only definitely positive results were accepted, one plus or doubtful reactions being regarded as negative. Furthermore, there may be a possibility of refining the antigen so that it can react with lesser amounts of antibody. However, in comparison with other bacterial diseases where complement fixation occurs, e. g., glanders or gonorrhea, the results are very favorable.

TABLE 2
THE RELATION BETWEEN COMPLEMENT FIXATION AND THE STAGE OF THE DISEASE

Case	Before Crisis	At Crisis	After Crisis (Number of days precedes result)
Epidemic typhus 1.....	Negative	5, + + M
Epidemic typhus 2.....	Negative	3, + + + M
Epidemic typhus 3.....	Negative	6, + + M; 16, + +
Epidemic typhus 4.....	Negative	4, negative; 14, negative
Epidemic typhus 5.....	Negative	9, negative
Epidemic typhus 6.....	3, + + M
Epidemic typhus 7.....	5, + + M
Epidemic typhus 8.....	3, + + M
Epidemic typhus 9.....
Epidemic typhus 10.....	Negative	4, negative
Epidemic typhus 11.....	1, negative
Endemic typhus 8.....	Negative	6, negative
Endemic typhus 10.....	Negative
Endemic typhus 12.....	Negative
Endemic typhus 13.....	Negative	7, + + + M
Endemic typhus 14.....	Negative	5, negative
Endemic typhus 15.....	Negative	2, negative; 7, + + M
Endemic typhus 16.....	4, negative; 7, negative
Endemic typhus 17.....	Negative	12, + + + M
Endemic typhus 18.....	Negative	6, + + + M
Endemic typhus 19.....	+ + + M	4, + + M
Endemic typhus 20.....	Negative	4, + + + + M
Endemic typhus 21.....	+ + M	5, + + + + M
Endemic typhus 22.....	+ + + + M	30, + + + + M; 64, + + + + ; 113, negative
Endemic typhus 23.....	Negative	3, negative
Endemic typhus 24.....	1, negative; 7, negative
Endemic typhus 25.....	+ + + + M	5, + + + + M
Endemic typhus 26.....	Negative	15, + + + + M
Endemic typhus 27.....	Negative	5, + + + + M
Endemic typhus 28.....	Negative	5, negative; 15, negative
Endemic typhus 29.....	5, + + + + M
Endemic typhus 30.....	Negative	2, negative; 9, + + + M
Endemic typhus 31.....	Negative	3, + + M; 42, negative
Endemic typhus 32.....	+ + M	3, + + M
Endemic typhus 33.....	7, + + M
Endemic typhus 34.....	Negative	6, + + + M
Endemic typhus 35.....	150, negative
Endemic typhus 36.....	+ + + + M	5, + + + + M
Endemic typhus 37.....	Negative	5, negative
Endemic typhus 38.....	Negative	5, + + M
Endemic typhus 39.....	Negative	5, + + M
Endemic typhus 40.....	Negative	6, + + + + M

M = mixed epidemic and endemic typhus bacilli antigen.

In regard to the stage of the disease in which positive reactions occur, remarkable observations were made. Twenty-five cases were tested at the height of the disease. Of these only two were positive, one a $++$ reaction, the other a $++++$. Both were tested one day before the crisis. The latter case had a negative, the former a positive blood culture.

Ten cases were tested at the crisis. Of these, two gave $++++$ reactions, one a $+++$, one a $++$, and the rest were negative. Thus we see a tendency towards an increased number of complement-fixing bodies at the crisis.

In the post-critical stage of the disease, the positive reactions are more common. In looking through Table 2, one will be struck by their number. Indeed, 71.8 percent are positive here, as was mentioned. One may trace the development in an individual case of a negative reaction at the height of the disease to a $++++$ reaction after the crisis.

It will be seen from this table that the complement-fixing antibodies reach their maximal concentration between the second and twelfth days after the crisis (endemic typhus Cases 15, 16, and 30). Their persistence has been studied in endemic typhus Case 22 (through the kindness of Dr. J. Reiss), in which a previously $++++$ reaction became negative between 64 and 113 days after the crisis, and in endemic typhus Case 31, in which there was a positive reaction three days after the crisis that was found to have disappeared thirty-nine days later. In one case, in which a diagnosis of typhus fever occurring five months previously was made on the patient's history, the reaction was negative (endemic typhus Case 35).

Complement Fixation in Control Cases.—In these studies, that most important fact in bacterial complement-fixation work was always kept in mind: The sum of the anticomplementary properties of antigen and serum, when mixed in a test, is greater than the sum of the anticomplementary activities of each by itself. Altho the reagents employed were of such degree of delicacy as to eliminate errors arising from anticomplementary action, the element of specificity was further enhanced by our considering such tests as gave a doubtful (\pm) or one plus reaction, negative. In fact, even such degree of reaction is regarded as negative for diagnosis in a Wassermann test based on one to four plus readings. This being the case, we may

safely say that all our non-typhus control cases gave negative reactions. (The great majority gave absolutely complete hemolysis; a few, a faint trace of inhibition in large amounts of serum.)

In all there were 104 control cases. These covered a great variety of pathologic conditions: acute febrile diseases such as typhoid fever, paratyphoid fever, influenza; chronic infectious processes such as lues, tuberculosis; new growths, surgical conditions, etc. In several of the acute febrile diseases in which the serum was tested during the febrile stage, a second test was made with the blood taken at some time in the apyrexial state. In all such instances, the results were likewise negative.

Complement Fixation in Typhus Fever Cases With Other Than Typhus Antigens.—As is known to serologists, certain sera, in the presence of any bacterial antigen, will bind complement. These reactions are absolutely non-specific, and one of the best examples we have is that of rabbit serum in large amounts. To avoid this element of non-specificity, the typhus antigen was titrated with normal, as well as with immune serum. Again, the typhus fever serum which gave positive results with typhus antigen was tested with the following bacterial antigens, the results being absolutely negative: Antigens made of *Bacterium coli*, *Staphylococcus aureus*, gonococcus, streptococcus (single antigens made of various types), and of some gram-positive, anaerobic bacilli having a morphology similar to that of the typhus bacillus (*Bacillus acnes* and bacilli isolated from ascitic fluid and from rabbit kidney). These antigens were prepared in the same manner as the typhus antigen.

We shall discuss later the relation of the results in complement fixation to other serologic results in typhus fever, as well as their significance in this disease.

AGGLUTINATION IN TYPHUS FEVER

The method of choice for performing the agglutination tests with typhus serum, or with the typhus organism, is the microscopic. This method is so well controlled that it equals the macroscopic for scientific accuracy. The reasons for employing the microscopic method rather than the macroscopic are these:

(1) The organism grows rather sparsely and requires strict anaerobic conditions; hence the large quantities necessary for suspensions as agglutinogens, are extremely difficult to obtain.

(2) The bacillus, without the addition of serum, has a marked tendency to clump on standing, making a twenty-four or forty-eight hour method impractical.

(3) On making comparative studies between the microscopic and macroscopic methods, we find that wherever we obtain a positive reaction with the former, we likewise obtain a positive result with the latter. Important to note is the fact that the macroscopic tests usually give positive readings in much higher dilutions than do the microscopic. Proagglutininoid zones are common in the former and indeed the clumping is best seen beyond the dilutions of 1:200, while proagglutininoid zones in the latter are never seen and the average limit of clumping is about 1:200.

(4) Furthermore, this method conserves the supply of serum, which at best is always limited in amount and which is needed for so many other studies.

The microscopic method which we apply consists in setting up a series of small test tubes, each containing an increasing dilution of the serum to be tested. Five tubes are ordinarily used. Tube 1 contains 0.1 c.c. of serum with 0.9 c.c. of 0.85 percent salt solution; Tube 2 contains 0.5 c.c. of the thoroughly mixed contents of Tube 1 plus 0.75 c.c. saline; Tube 3, 0.5 c.c. of contents of Tube 2 plus 0.5 c.c. saline; Tube 4 contains 0.5 c.c. of contents of Tube 3 plus 0.5 c.c. saline and Tube 5, 0.5 c.c. of contents of Tube 4 with 0.5 c.c. saline. Thus accurate dilutions (each in a test tube) of 1:10, 1:25, 1:50, 1:100, and 1:200 are made. The serum used is inactivated at 56 C. for one-half hour.

The agglutininogen is made by suspending the growths of several strains of typhus bacilli in saline solution. This is then shaken, either by hand or in the shaking machine, until the clumps are thoroughly broken up. It is then centrifuged gently for a short time so as to clear the suspension of any small masses or clumps. The accuracy of the final dilution (that of the serum mixed with agglutininogen) is maintained by using the same loop for each of these reagents and taking off a drop from the surface of each fluid. In this way the final dilutions of 1:20, 1:50, 1:100, 1:200, and 1:400 are made.

The tests are read at the end of one hour at room temperature. When all the bacteria are clumped together into solid masses with clear spaces between such clumps, the reaction is called ++; when the clumps are definite, but not as large in size as in the ++ instance, the reading is considered +; and where there is distinct agglutination, but between the masses a few unclumped bacteria, the reaction is called slight. Care is taken to examine the entire area to avoid the confusing clumping which usually occurs about the periphery of any drop of emulsion of bacteria.

As is seen from the foregoing, the readings are very conservative. In view of the short time (one hour) allowed for the reaction (in comparison with macroscopic tests, in glanders, for example, in which the readings are made at the end of 72-96 hours) we have a sufficient explanation for the average agglutination titer of 1:200 in typhus fever sera.

Furthermore, rabbit immune serum of high potency (demonstrated by the presence of precipitins and complement-fixing bodies in high dilutions) shows an agglutination titer varying between 1:400 and 1:800, when the tests are made in a similar manner. One would expect therefore that natural immune serum would give a lower titer, coinciding possibly with our findings of 1:200 in convalescent typhus fever cases.

Agglutination During the Course of Typhus Fever.—Here, as well as in our complement-fixation work, we dealt with two distinct strains of typhus bacilli at the inception of the work, the organism obtained from epidemic typhus fever and the one from endemic typhus fever.

Comparative experiments demonstrated that an epidemic typhus agglutinin gave clumping with serum from endemic typhus fever as well as from epidemic typhus fever, and that an endemic typhus agglutinin likewise gave positive agglutination with serum from patients suffering from either form of typhus fever (Table 3).

TABLE 3
THE RELATION BETWEEN ENDEMIC TYPHUS AGGLUTININ AND EPIDEMIC TYPHUS AGGLUTININ

Case	Endemic Typhus Agglutinin					Epidemic Typhus Agglutinin				
	1:20	1:50	1:100	1:200	1:400	1:20	1:50	1:100	1:200	1:400
Endemic typhus 3.....	+	+	+	Slight	—	++	++	++	+	—
Endemic typhus 7.....	+	+	Slight	Slight	—	—	—	—	—	—
Endemic typhus 9.....	Slight	Slight	—	—	—	+	+	—	—	—
Endemic typhus 8.....	++	++	++	+	—	++	++	—	—	—

These results are somewhat irregular. One would expect the typhus agglutinin to react more powerfully in the three epidemic typhus cases than it did, but one should also consider that the strains used were freshly isolated while the endemic typhus strains were older. However, the cross agglutination between epidemic and endemic typhus fever is well illustrated.

In applying a mixed agglutinin, i. e., one containing strains from both epidemic typhus fever and endemic typhus fever, positive agglutinations were obtained in more instances than positive complement fixations. In making up the tables, I have considered such reactions as occur in dilutions of 1:50 or higher as positive, for reasons which I shall give later.

Table 4 shows that in forty-three typhus cases (including the endemic form), during some period in the disease, thirty-nine, or 90.7 percent, were positive. Of these cases investigated, twenty-four were tested at the height of the disease. Here, all were negative but two, endemic typhus Cases 20 and 39, both having a titer of 1:100. In both cases, the blood was tested a day before the crisis occurred. Ten cases were tested on the day of the crisis; of these, seven were negative and three positive. In the apyrexial period, thirty-eight were studied; only three were negative, and 92.1 percent were positive. Hence we see, just as in our complement fixation work, that the agglutinins are

usually absent at the height of the disease, but increase in amount at the crisis until they reach their maximum well along in the apyrexial period. In epidemic typhus Case 5, there was no agglutination four days after the crisis, but ten days later the serum agglutinated at 1:50. In endemic typhus Case 8, while there was a negative reaction one day after, there was a positive reaction (1:200) six days after the crisis. Also in endemic typhus Case 15, a negative reaction two days after the crisis became 1:200 five days later.

TABLE 4
AGGLUTINATION DURING THE COURSE OF TYPHUS FEVER

Case	Height of Disease	Crisis	After Crisis (Number of days precedes result)
Epidemic typhus 1.....	—	Negative	
Epidemic typhus 2.....	Negative	—	5, 1:100 slight
Epidemic typhus 3.....	Negative	—	3, 1:200 +
Epidemic typhus 4.....	Negative	—	6, 1:100 +
Epidemic typhus 5.....	Negative	—	4, negative; 14, 1:50 +
Epidemic typhus 6.....	—	—	9, 1:100 +
Epidemic typhus 7.....	—	—	3, 1:200 +
Epidemic typhus 8.....	—	—	5, 1:100 slight
Epidemic typhus 9.....	—	—	3, 1:100 slight
Epidemic typhus 10.....	Negative	—	
Epidemic typhus 11.....	—	—	4, 1:100 +
Endemic typhus 8.....	Negative	—	1, negative; 6, 1:200 +
Endemic typhus 10.....	Negative	—	6, 1:100 +
Endemic typhus 12.....	—	Negative	
Endemic typhus 13.....	—	Negative	7, 1:200 +
Endemic typhus 14.....	Negative	—	5, negative
Endemic typhus 15.....	Negative	—	2, negative; 7, 1:200 +
Endemic typhus 16.....	—	—	4, 1:200 +; 7, 1:200 +
Endemic typhus 17.....	—	—	4, 1:200 +
Endemic typhus 18.....	—	Negative	4, 1:200 +
Endemic typhus 19.....	—	1:350 slight	
Endemic typhus 20.....	1:100 +	—	4, 1:100 +
Endemic typhus 21.....	Negative	—	5, 1:50 +
Endemic typhus 22.....	Negative	1:100 +	30, 1:1000 +; 64, 1:1200 +
Endemic typhus 23.....	Negative	—	113, 1:200 slight; 154, 1:50 +
Endemic typhus 24.....	—	—	3, 1:200 +
Endemic typhus 25.....	Negative	—	1, 1:200 +; 7, 1:200 +
Endemic typhus 26.....	Negative	—	5, 1:1400 +
Endemic typhus 27.....	Negative	—	15, 1:800 +
Endemic typhus 28.....	Negative	—	5, negative
Endemic typhus 29.....	—	—	1, 1:100 slight
Endemic typhus 30.....	Negative	—	5, 1:500 +
Endemic typhus 31.....	Negative	Negative	2, 1:100 +; 9, 1:100 +
Endemic typhus 32.....	Negative	1:800 +	3, 1:100 +
Endemic typhus 33.....	—	—	3, 1:200 +
Endemic typhus 34.....	Negative	—	7, 1:200 slight
Endemic typhus 35.....	—	—	6, 1:200 +
Endemic typhus 36.....	—	Negative	150, 1:500 +
Endemic typhus 37.....	Negative	—	5, negative
Endemic typhus 38.....	—	Negative	5, 1:100 slight
Endemic typhus 39.....	—	—	5, 1:100 slight
Endemic typhus 40.....	1:100 +	—	5, 1:100 +
Endemic typhus 40.....	Negative	—	6, 1:200 slight

Daily agglutination tests (for some of which we are indebted to Dr. A. Brodey) performed on endemic typhus Case 15, showed that there were no agglutinations until four days after the crisis, when

the titer was 1:50. The next day it rose to 1:100 and for seven days in the apyrexial stage was 1:200, the level at which it remained. Likewise in endemic typhus Case 17, daily tests showed that no agglutinins were present until the fourth day after crisis, when the titer reached 1:200. We may conclude then that the maximal concentration of agglutinins appears about four days after the crisis.

In regard to the persistence of agglutinins, we may state that they are demonstrable at least five months after the crisis. Endemic typhus Case 35* showed a reaction of 1:500 five months after the crisis. Endemic typhus Case 22, during varying periods after the crisis, showed the following reactions:

Days after Crisis	Reaction
30	1:1000
64	1:1200
113	1:200
154	1:50

In this case there was a positive complement fixation (+ + + +) on the sixty-fourth day, but a negative reaction thereafter. It is probable, then, that complement-fixing bodies disappear much more quickly than the agglutinins.

Agglutination Tests on Control Cases.—A great many of the control cases were tested both for complement fixation and agglutination. There were forty-four cases in this series, and the diagnosis included a great variety of conditions. In seven of these cases, there occurred agglutination in a dilution of 1:20, but none in higher dilutions. Of the seven cases, one was a brain tumor; two, valvular defects; two, general paresis; one, carcinoma of the tonsil; and one a case of alcoholism. In all, complement fixation was negative. It is on this account that in the tabulations I regarded only such results positive as gave agglutination in dilutions of 1:50 or higher.

These non-specific reactions in low dilutions are not peculiar to typhus fever. It is now generally accepted that traces of antibodies may be found in normal serum, but in immune serum these are increased to greater or less degrees. Or, in the foregoing instance, when the dilutions are so low, "serum action" (probably through a colloid phenomenon) is the cause of the clumping.

* This patient was in a debilitated condition for five months after an attack of supposed typhoid fever, lasting ten days, accompanied by severe headache. As it did not seem likely that an attack of typhoid fever of such short duration would so weaken an individual, the possibility of typhus fever was entertained by Dr. Libman, who referred the case to us for serologic study.

Besides those mentioned, there was another series of twelve cases. These were sent to us for diagnosis, and subsequent investigation proved that none of the cases was typhus fever. All these showed negative agglutination reactions. All cases were febrile and included such conditions as typhoid fever, meningitis, tuberculosis, influenza, etc. In these the blood was tested both during the febrile stage and in the post-critical period, with the same negative results.

A third series of controls comprised forty cases. Of these, three cases gave positive results. Case 1 was an adult male who was suffering from cellulitis of the hand at the time the test was made. He gave a history of having served in an army corps stationed at the Mexican border some years ago, and at that time he had been seized with a severe illness, for which he had been treated at the camp hospital. Because of his incipient paresis, he was unable to give a detailed account of his illness. The other two cases were immigrants living in the slums. Because of their lack of intelligence, a proper previous history could not be obtained. The first case showed agglutination of 1:100; the other two, 1:200. Complement-fixation tests in all three were negative. It is reasonable to suppose that these three cases (from a total of ninety-six controls tested) had had, at one time or another, typhus fever.

Agglutination With Other Than Typhus Organisms.—During the course of this work, I have endeavored to use as agglutinogens all such bacteria as we were able to obtain, in cases other than typhus fever, that had in any way some resemblance morphologically to the typhus bacillus. In all such instances, when the agglutininogen was mixed with convalescent typhus serum, the results were negative. For instance, in the case of *Bacillus acnes* (for several strains of which we are indebted to Dr. E. P. Bernstein, Lebanon Hospital, and to Dr. C. E. A. Winslow, Museum of Natural History, New York City), one of our reports shows:

Dilutions of Immune Serum	Agglutininogen	
	<i>Bacillus Acnes</i>	<i>Bacillus Typhi-Exanthematici</i>
1: 20	Negative	++
1: 50	Negative	++
1: 100	Negative	++
1: 200	Negative	++
1: 400	Negative	+
1: 500	Negative	+
1: 800	Negative	+

PRECIPITIN IN TYPHUS FEVER

We have demonstrated that in serum from typhus fever convalescents there are present specific precipitins which react with filtrates of the typhus bacilli. Our attention was first called to the fact that precipitins may be present by the appearance of a precipitate in immune rabbit serum which was kept in the cold. This rabbit had had injected large doses of bacteria and the blood, when withdrawn, contained possibly precipitogen and precipitin at the same time.

METHODS

The precipitogen was prepared by inoculating 2 percent glucose broth with typhus bacilli and allowing cultures to grow one month. Different strains were used so as to give a polyvalent precipitogen. The cultures were inserted into Buchner tubes or Noguchi tubes, and the media, in such cases, covered over with sterile liquid petrolatum. No serum was used in these cultures. The sediment or growth of each tube having been stained to be certain of its purity, the entire material was then filtered through a Berkefeld filter. The clear filtrate was employed as precipitogen. We have tried the method of Besredka of precipitating the protein of the bacterial cells, drying, and subsequently suspending the residuum in saline or water, but the former method is more advantageous. The serum used was cleared and inactivated. Dilutions were made at the beginning of the work of 1:100, 1:500, 1:1000, 1:1500, and 1:5000. Control tests consisted in using an immune (rabbit) serum of known titer and a known negative serum (from a case not having typhus fever). The test was performed by adding 1 c.c. of precipitogen to 0.5 c.c. of each dilution in individual test tubes. The results were read at the end of two hours at 37 C. When no reaction was noticed at the end of this time, such tests were placed in the ice box over night. Care must be exercised in reading reactions in over night tests, for precipitations may occur which are due to contaminations, usually with *Bacillus subtilis*. Subcultures should be made to eliminate such errors. However, most of our tests were read at the end of two hours at 37 C. Some showed clouding in low dilutions almost immediately on the addition of the precipitogen.

After we had obtained several positive results, we simplified the method and employed only six tubes. Tube 1 contained 0.05 c.c. of suspected serum; Tube 2 contained 0.01 c.c. of suspected serum; Tube 3 contained 0.5 c.c. of suspected serum; Tube 4 contained 1.0 c.c. of suspected serum; Tube 5 contained 0.05 c.c. of immune serum; Tube 6 contained 1.0 c.c. of control (negative) serum. In all tubes, 2 c.c. precipitogen were added and sufficient saline to equalize to 3 c.c. Readings were made at the end of two hours at 37 C.

Precipitin During the Course of Typhus Fever.—There is considerable resemblance in the results obtained in investigating precipitin to those obtained in the study of agglutinin and complement-fixing antibodies.

Of ten cases studied at the height of the disease, all were negative. Three cases were tested in which the blood was withdrawn at the crisis. Of these one showed distinct clouding in 1:1000 dilution. Of the nineteen cases tested in the apyrexial period, fourteen, or 73.6 percent, gave precipitations. Three of these reacted in dilutions of 1:1500; four in dilutions of 1:1000, while the rest showed precipitates in dilutions of 1:100 (Table 5).

TABLE 5
PRECIPITIN DURING THE COURSE OF TYPHUS FEVER

Case	Height of Disease	Crisis	After Crisis (Days after crisis precedes results)
Epidemic typhus 2.....	5, 1:100
Endemic typhus 8.....	Negative
Endemic typhus 10.....	Negative	6, 1:100
Endemic typhus 12.....	Negative
Endemic typhus 13.....	Negative	7, 1:100
Endemic typhus 14.....	Negative
Endemic typhus 15.....	7, 1:100
Endemic typhus 16.....	7, negative
Endemic typhus 17.....	7, 1:1000
Endemic typhus 18.....	4, 1:100
Endemic typhus 22.....	30, 1:1500
Endemic typhus 24.....	1, negative; 7, 1:1500
Endemic typhus 25.....	Negative
Endemic typhus 26.....	Negative	15, 1:1000
Endemic typhus 27.....	Negative	5, 1:1500
Endemic typhus 28.....	Negative	5, negative
Endemic typhus 29.....	Negative	5, 1:1000
Endemic typhus 30.....	2, negative; 9, negative
Endemic typhus 31.....	3, negative
Endemic typhus 32.....	3, 1:1000
Endemic typhus 34.....	Negative	6, negative
Endemic typhus 38.....	1:1000
Endemic typhus 39.....	Negative	5, 1:100
Endemic typhus 40.....	Negative	6, 1:100

Table 5 shows that precipitin is absent at the height of the disease but becomes in evidence at the crisis. Thence it proceeds to increase until well along in the post critical stage (see endemic typhus Case 24: one day after crisis, no precipitation; seven days after, positive 1:1500).

With each test, as was stated, a negative serum was included. Of numerous such control cases, none has shown a positive reaction. This is in accord with our knowledge of precipitins, since these bodies are regarded as highly specific.

OTHER ANTIBODIES IN TYPHUS FEVER

Bacteriolysins (Bacteriocidins).—We could not determine in typhus fever immune serum the presence of bacteriolysins or bacteriocidins against the typhus bacillus. This does not signify that no such anti-

bodies exist, but that, if present, we have not as yet been able to devise a method for their detection. The reasons for this are:

(1) Animal experimentation is unsuitable. The organism rapidly loses its virulence, hence the protective power of immune serum can not be studied, since the control animals (those injected with bacteria alone) are not affected.

(2) In vitro, bacteriolysin experiments are equally without result. We have tried the plate method, using serial dilutions of immune serum (to obviate the complement deviation of Neisser-Wechsberg), as well as a capillary pipette method, but in all experiments the control plate (i. e., plate poured from a mixture of organisms and saline alone) would show no growth, or, at the utmost, one or two colonies. We could not maintain in our plate experiments the strict anaerobiosis that the organism requires.

TABLE 6
THE OPSONIC VARIATIONS IN ENDEMIC TYPHUS, CASE 17

Day of Disease	Average Number of Bacteria Taken up by Phagocytes in		Index
	Pooled Normal Sera	Typhus Serum	
1 day before crisis.....	2.0	1.8	0.9
Crisis	3.2	3.2	1.0
1 day after crisis.....	5.0	5.5	1.1
2 days after crisis.....	5.4	7.0	1.4
3 days after crisis.....	2.9	4.2	1.49
4 days after crisis.....	1.5	2.2	1.5
5 days after crisis.....	2.5	3.2	1.47
6 days after crisis.....	1.7	2.2	1.3
7 days after crisis.....	1.2	2.4	2.0

(3) We have attempted to reproduce Pfeiffer's phenomenon in guinea-pigs, using typhus immune pigs and typhus bacilli, but there was no bacteriolysis as in cholera. In this respect, if there are no bacteriolysins present, typhus fever would form no exception, since it is well known that the organisms of the hemorrhagic septicemia group, altho their immune sera contain complement-fixing bodies, yet show no bacteriolysins. Numerous experiments were made in which bacteria were suspended in serial dilutions of immune serum, but here, as well, no lysis of the typhus bacilli was noted. In all instances the organisms became gram-negative, but the same change was noted with normal serum controls. However, we do not wish to state definitely, on the basis of this series of experiments, that there are bacteriolysins present

in typhus immune serum unless we can substantiate the results with animal investigations, using a virulent strain of the organism.

Opsonins.—That immune opsonins against this organism are present in typhus immune serum is quite evident. In testing for opsonins, Wright's method was used with inactivated serum.

While discrepancies are common with this method (and such have occurred here, when two workers repeated the same test), yet the average results demonstrate that the opsonic index increases at the crisis and remains high in the convalescent stage of the disease. An example of this will be seen by reference to Table 6, endemic typhus Case 17. In this case, all tests were made by the same individual.

Similar rises were noted in artificially immunized serum (rabbit). The probability is that this method is one of the most potent, on the part of the patient, in overcoming the infection. We noted, during these examinations, that bacteria taken up by phagocytes had a marked tendency to become fragmented and take the stain very lightly. Lysis of the typhus bacilli apparently is very active within polymorphonuclear cells of typhus immune subjects.

Other features of this work, the occurrence of allergic reactions (using extracts of typhus bacilli as allergens) and the nature of the toxin produced, which are in progress now, will be reported later.

Having thus demonstrated the presence of various antibodies in typhus fever serum, I shall now discuss certain phenomena depending upon these facts.

THE IDENTITY OF THE ORGANISM ISOLATED FROM CASES OF ENDEMIC TYPHUS FEVER WITH THAT ISOLATED FROM CASES OF EPIDEMIC TYPHUS FEVER

It was my first endeavor to ascertain the serologic relation of the endemic strain of the typhus bacillus to that isolated from epidemic typhus fever. This was studied by cross fixation, both in artificially produced immune serum and in natural immune serum, and similarly by cross agglutination.

Artificially produced immune serum was prepared by the method previously described. Rabbits were immunized both to epidemic typhus fever strains and to an endemic strain, for at that time there were available five strains of epidemic typhus fever organisms (A, S, 1, 3, 4) and but one from endemic typhus (B 40). Individual

antigens were likewise prepared, one containing none but epidemic typhus strains, the other, the endemic strain.

Table 7 presents the results of experiments in cross fixation. From this it will be seen that the bacteria isolated from epidemic typhus fever and those from endemic typhus fever are merely two strains of the same organism.

TABLE 7
EXPERIMENTS IN CROSS FIXATION WITH RABBIT IMMUNE SERUM

(a) Titrations with Epidemic Typhus Strains Antigen						
Amount of serum.....	0.0005	0.001	0.005	0.01	0.05	0.1
Epidemic typhus immune serum....	+	+	+	+	+	+
Endemic typhus immune serum.....	0	±	+	+	+	+
(b) Titration with Endemic Typhus Strain Antigen						
Amount of serum.....	0.0005	0.001	0.005	0.01	0.05	0.1
Epidemic typhus immune serum....	±	+	+	+	+	+
Endemic typhus immune serum.....	0	+	+	+	+	+

The sign + = inhibition of hemolysis; ± = incomplete inhibition of hemolysis; 0 = hemolysis.

TABLE 8
EXPERIMENTS IN CROSS AGGLUTINATION WITH IMMUNE RABBIT SERUM
(a) Using Endemic Typhus Strain Immune Serum

Serum Dilution	Epidemic Typhus Strains Agglutinin	Endemic Typhus Strain Agglutinin
1: 20	+	++
1: 50	+	++
1: 100	+	++
1: 200	+	++
1: 400	Slight	+
1: 500	Negative	+
1: 600	Negative	Slight

(b) Using Epidemic Typhus Strains Immune Serum

1: 20	++	+
1: 50	++	+
1: 100	+	+
1: 200	+	Slight
1: 300	Negative	Negative

In the case of natural immune serum, as we have it in convalescent epidemic typhus fever patients, the same phenomenon is to be noted. A comparison with Table 1 will show that endemic typhus immune serum has the same specific complement-fixing bodies as epidemic typhus fever immune serum.

Agglutination proves the same point, namely, that the specific agglutinin developed in epidemic typhus immune serum is the same as that in endemic immune serum. This could be demonstrated in artificially produced immune serum as well as in natural (Tables 3 and 8). In the artificially produced immune serum, the agglutinin

that corresponds to the strain used in immunization gives a higher titer, which is to be expected.

We therefore conclude that from a serologic viewpoint, both these types, the organism obtained from endemic and the one from epidemic typhus fever, are two strains of the same bacterium.

SEROLOGIC STUDIES IN ANIMALS

Monkeys.—It was the practice to test the serum of monkeys, before use, for complement-fixing bodies, as well as agglutinins. In this way, not only could the subsequent reactions be studied more accurately, but we also learned that the average normal monkey has no such antibodies present. In the experiments with eight normal monkeys, complement fixation, as well as agglutination, was negative.

Experimental typhus fever in the monkey gave rise to serologic changes in the same manner as in human beings, as will be seen by consulting Table 9. Hence, of six monkeys that reacted to the typhus virus, complement-fixation and agglutination tests were positive in five, or 83.3 percent. These reactions were still positive twenty days after the crisis.

TABLE 9
COMPLEMENT FIXATION AND AGGLUTINATION IN MONKEYS

Number of Monkey	Date of Injection of Virus	Duration of Reaction	Complement Fixation	Agglutination	Number of Days After Crisis Tested
1	Jan. 7	Jan. 7-20	++++	1: 200	8
2	Jan. 29	Feb. 5-12	++	1: 100 (slight)	20
3	Jan. 21	Jan. 29-Feb. 5	Negative	Negative	27
7	Feb. 5	Feb. 13-21	++	1: 100	8
10	Jan. 21	Feb. 5-13	++	1: 100 (slight)	11
11	Jan. 29	Feb. 5-11	+++	1: 100	13

The effect of repeated injections of monkeys with avirulent, living, non-sensitized typhus bacilli, was investigated. In animals in which previous reactions were negative, large doses were required before there was any noticeable change in the serum content of antibodies.

Monkey 2 was injected with living, avirulent vaccine of typhus bacilli on October 8, 12, and 17, in doses of two billion, four billion, and four billion, respectively. October 18, its blood showed a ++ complement fixation, but a negative agglutination. Of course, in this instance, there was a short interval between the last injection and the withdrawal of the blood. However, this vaccination was continued for three additional injections with dosages running up to eight billion. Five days after the last injection, the complement-fixation test showed + + + +, and the agglutination titer was 1: 500.

Monkey 1 likewise received six injections. Nine days after the last injection, blood was withdrawn and tested. Complement fixation showed + + + + and the agglutination titer was 1:100.

Monkey 3 showed the same results with five injections, dosages varying from four to six billion.

Monkey 6 after six injections of from three to ten billion dosage showed, nine days after the last injection, a complement fixation of + + + + and an agglutination of 1:500.

However, in such "vaccinated" animals, these reactions may disappear after a time.

Monkey 2, on November 3, 1914, showed a + + + + complement fixation and 1:500 agglutination. On January 3, 1915 (two months later), the reactions were negative. Monkey 3, on the former date, gave a + + + + complement fixation and 1:100 agglutination. Two months later these likewise disappeared. Monkeys 1 and 6 retained their reactions, altho somewhat weaker in degree, during this time.

As the agglutination in typhoid fever is no absolute sign of protection from that disease, so in typhus fever, complement fixation and agglutination, while often appearing in immunity, are no indications of immunity. The four monkeys mentioned, two with reactions and two without, were injected with active typhus virus. Three monkeys (1, 2, and 3) did, while one did not, react (6). Of the three showing typical typhus fever, one had given complement fixation and agglutination previously to being inoculated with the virus (Monkey 1), while two had been without such manifestation (Monkeys 2 and 3). Hence, in spite of the presence of complement-fixing bodies and agglutinins, experimental typhus fever could be produced. In two of these monkeys, the reactions reappeared after the crisis had occurred.

The production of complement-fixing bodies as well as agglutinins in monkeys can be paralleled in man. In regard to the latter, in one case, four injections were given, at intervals of five days, of 100, 250, 500, and 1,000 million killed, non-sensitized bacteria. Nine days after the last injection, the reactions were negative. In another case (Dr. H. Plotz, Dr. A. Loewenthal, and Dr. A. Brodey volunteering their services to act as subjects for vaccination), five injections were given subcutaneously at five-day intervals, of 250, 500, 1,000, 1,500, and 2,000 million killed, non-sensitized bacteria. Nine days after the last injection, the complement fixation was + + + +, while the agglutination titer was 1:500.

The results in prophylactic vaccination against typhus fever, a work which is now in progress, will be reported later.

Guinea-Pigs.—The results of the serologic investigations in typhus-immune guinea-pigs were quite different from those in other animals.

Guinea-pig 4 was injected with blood from typhus Case 3 on April 14, 1914, and reacted April 22. On June 16, it was again injected with a guinea-pig typhus virus. There was no reaction, hence the animal was proved immune. This animal was bled July 6, and the serum was then studied. Normal guinea-pig serum was used for control.

Altho the animal was proved immune, yet the serum contained no agglutinin or complement-fixing bodies. The same results were obtained on repetition of this experiment. It is most probable that the guinea-pig reacts to the typhus virus and develops subsequently a high grade of immunity by means of its tissue elements and only to a very slight degree by means of the circulating blood. In this respect, there is the analogous tolerance of rats to diphtheria toxin, altho no circulating antitoxin is demonstrable.

There is another possibility in that the opsonins may exert great influence in these animals and likewise add to the factors of the cellular immunity which may be present. When guinea-pig leukocytes and inactivated immune serum were used, the latter gave much higher indices than normal, inactivated guinea-pig serum.

Rabbits.—Rabbits are not susceptible to the typhus bacillus in small amounts. If given intravenously in large doses, they will succumb in 3-7 days. In such animals, the autopsy reveals no distinct pathologic changes. The animals do not develop a carrier state, as they do in the case of typhoid bacilli, but die most likely from toxemia.

When small yet increasing amounts of bacteria are given, rabbits develop very potent immune serum. I shall illustrate the serum studies on the rabbit by giving a report of one of the typically reacting rabbits.

Rabbit B was injected on May 26, 30, June 3, and 7, 1914, with one-half the growth on a slant, the full growth on one, two, and three slants, respectively. The first two injections were with killed bacteria, the second two with live organisms. Since the growth on a slant is not very luxuriant, these quantities represent fewer organisms than one might presuppose. As many different strains as possible were used to prepare immune serum. Nine days after the last injection, June 16, 1914, the rabbit was bled from the internal jugular vein and in that manner serum obtained. The serum in this instance showed: Complement fixation + + + + with 0.0005 c.c. serum; agglutination 1:500 +; precipitation 1:2500 +; opsonic index (compared with non-immunized rabbits) 3.0. Unfortunately, the bactericidal experiments were impossible for the reasons stated. This immune serum, however, produces no lysis of the bacteria when the former is added to the latter in vitro.

When such immune serum was titrated against different strains as antigens, it was found that all strains cross-fixed to a greater or less degree, but that there were appreciable differences between epidemic typhus and endemic typhus strains. In other words, the typhus bacillus is divided into two distinct strains: the epidemic typhus and the endemic typhus strains.

SEROLOGIC REACTIONS IN INDIVIDUALS AFTER EXPOSURE TO TYPHUS FEVER

Observations made upon the serum of two individuals proved very interesting. In both, there was a definite exposure to the typhus virus: one (Dr. B.) to the endemic and epidemic types; the other (Miss L.) to the epidemic form only. At no time did either of them have any symptoms which were recognizable as due to typhus fever. Yet they showed complement-fixing antibodies and agglutinins in this manner:

Date	Complement Fixation Test	Agglutination Test
	Dr. B.	
Dec. 15, 1914	+	1: 500 +
Dec. 21, 1914	+	1: 200 +
March 1, 1915	+	1: 50 +
April 17, 1915	Negative	1: 20 +
	Miss L.	
April 17, 1915	+	1: 200 +

The significance of these reactions will be discussed in the following section on experimental studies in typhus fever.

THE RELATION BETWEEN THE SEROLOGIC RESULTS AND TYPHUS FEVER. CONCLUSIONS

There is a most intimate relation between the typhus bacillus and typhus fever. The serologic reactions occur in an orderly manner. Complement-fixing bodies are usually not seen at the height of the disease, but are demonstrable as the crisis is attained and increase in concentration in the post critical, afebrile state; the same is true of agglutinins, as well as precipitins. These antibodies are developed in the body as the result of an antigen circulating at the height of the disease. They are demonstrated *in vitro* when the organism isolated from the blood of typhus fever patients is used as artificial antigen. Hence, but one conclusion is possible, typhus fever is a reaction against this organism.

Altho these reactions occur in coincidence with the process of immunity, they are merely signs which usually appear in immunization, but are not indicators of the existence of absolute immunity.

In so far as these reactions relate to the results of the blood cultures in typhus fever, these facts were noted:

There were sixteen cases in which the blood culture was taken at the height of the disease and in which bacteria were not found. In Table 10, it will be seen that most of our strongest serologic reactions occurred in just such cases.

TABLE 10
THE RELATION BETWEEN NEGATIVE BLOOD CULTURES AND SEROLOGIC REACTIONS

Case	Result of Blood Culture	Complement Fixation	Agglutina- tion	Precipita- tion
Epidemic typhus 9.....	Negative	++	1:100	Not done
Endemic typhus 8.....	Negative	++++	Negative	Positive
Endemic typhus 12.....	Negative	Negative	Negative	Negative
Endemic typhus 14.....	Negative	Negative	Negative	Not done
Endemic typhus 15.....	Negative	++	1:200	Positive
Endemic typhus 17.....	Negative	++++	1:200	Positive
Endemic typhus 19.....	Negative	++++	1:350	Not done
Endemic typhus 21.....	Negative	++++	1:50	Not done
Endemic typhus 22.....	Negative	++++	1:1200	Positive
Endemic typhus 25.....	Negative	++++	1:1400	Not done
Endemic typhus 26.....	Negative	++++	1:800	Positive
Endemic typhus 27.....	Negative	++++	Negative	Positive
Endemic typhus 28.....	Negative	Negative	1:100	Negative
Endemic typhus 30.....	Negative	+++	1:100	Negative
Endemic typhus 31.....	Negative	++	1:100	Negative
Endemic typhus 37.....	Negative	Negative	1:100	Not done

There are two exceptions in the endemic typhus Cases 12 and 14. In both these, not only the blood culture, but all the serologic reactions are negative. Here the suspicion arises as to whether they were really cases of endemic typhus fever. However, they were included in our records as the clinical picture resembled endemic typhus fever.

In cases in which the blood cultures were positive (twenty cases), there were only four instances where the complement fixation showed +++++, and in no case was the agglutination titer higher than 1:200. There is apparently no relation in the degree of the serologic reactions to the number of colonies isolated in positive blood cultures.

The presence of these antibodies to the typhus bacillus having been shown, and also their manner of production and their relation to typhus fever, as well as their absence in cases other than epidemic or endemic typhus fever, the connection between this organism and typhus fever becomes apparent.

III. EXPERIMENTAL STUDIES*

GEORGE BAEHR, HARRY PLOTZ, AND PETER K. OLITSKY

In this section, we shall confine ourselves to a report of that part of the experimental work on typhus fever which has a bearing upon the etiology of the disease. The work upon the artificial production of immunity is still in progress, and except for those observations which possess some etiologic significance, its presentation will be reserved for a future communication.

The results of the investigation which forms the basis of this report have supplemented the findings of the bacteriologic and serologic investigations, and have supplied additional methods of proof that the organism discovered by Dr. Plotz¹⁰⁵ in 1914 is the etiologic agent in typhus exanthematicus.

The experiments were chiefly carried out upon monkeys and guinea-pigs, 13 macacus rhesus monkeys and about 350 guinea-pigs being used. (The active assistance of Dr. A. Brodey and Dr. H. Zamkin during the course of the work was of great value and is much appreciated.) For the purpose of propagating the various strains of typhus virus—a term used throughout this paper to designate infectious blood of an individual or an animal with typhus fever in contradistinction to the bacillus itself—and for much of the experimental work, guinea-pigs proved as satisfactory as monkeys, an experience which coincided with that of Anderson and Goldberger² and other workers⁹².

For part of the work, eight strains of typhus virus were used. Six were obtained through the kindness of Dr. J. J. O'Connell from individuals with epidemic typhus fever who entered the port of New York after the Balkan Wars, and two from patients in the wards of the Mount Sinai Hospital who were suffering from the local endemic typhus known generally in the United States as Brill's disease^{24, 25}. One of the latter strains was sent to us by Dr. Anderson, who had originally obtained it from a case in the wards of the Mount Sinai Hospital on September 20, 1911,⁶ and had kept it alive since that time by repeated passages through monkeys and guinea-pigs. We were readily able to confirm his observation² that during that time it had undergone no apparent change in its virulence, the disease produced in animals by its inoculation being indistinguishable from that which

*This work was done under the tenure of the Eugene Meyer, Jr., and Moses Heineman Fellowships in Pathology.

followed the inoculation of any other typhus virus after its first passage through a guinea-pig.

Before entering into the details of the experimental work, a brief survey of the most important results of previous investigations in this field is advisable.

That the virus of typhus fever is present in the circulating blood during the febrile period of the disease was discovered by Moczutkowski,⁸¹ who in 1876 inoculated himself with the blood of a typhus patient and eighteen days later developed the disease. Nicolle⁹⁰ first succeeded in inoculating a chimpanzee with the disease, and subsequently was able to transfer the infection from the chimpanzee to the macacus sinicus monkey. The macacus rhesus monkey was first proved to be susceptible to the direct inoculation of human virus by Anderson and Goldberger.¹⁶ And the susceptibility of the guinea-pig to the disease was discovered by Ricketts and Wilder.¹¹⁷

These workers found that inoculation of an animal with an active typhus virus is succeeded by an incubation period usually of seven to fourteen days' duration, occasionally somewhat longer, after which the animal runs a febrile course, usually lasting from four to eleven days. During this time the temperature ranges between 104 and 106 F. (40 and 41 C.) and the blood is usually infective for other animals.

After the defervescence of the fever by crisis, or rapid lysis, the animals were found to possess an immunity to the disease, which, in one monkey tested by Anderson,² lasted at least two and a half years. Anderson and Goldberger¹⁶ likewise demonstrated that animals which have recovered from the Mexican typhus fever are also immune to the virus of Brill's disease, and vice versa, thereby indicating the relationship, tho not the identity, of the two diseases.

That the louse can act as the intermediary host in the transfer of typhus virus from man to monkey and from monkey to monkey has been proved independently by the experiments of Nicolle, Compte, and Conseil,⁹⁴ Ricketts and Wilder,¹¹⁸ and Anderson and Goldberger.¹⁶ And recently, Sergeant, Foley, and Vialatte¹²³ have used lice to effect a transfer of virus from man to man.

These are the main facts which have been determined up to date by experimental research. The first point for us to ascertain was whether the organism recovered from the blood of typhus fever patients was also found in animals in which the disease had been produced by the inoculation of typhus blood.

BLOOD CULTURES ON ANIMALS WITH TYPHUS FEVER

For this purpose a series of twenty-four guinea-pigs was inoculated with 2.5-4 c.c. of defibrinated blood obtained from patients or animals with typhus fever and diluted with an equal volume of normal salt solution. The blood cultures were then made sometime after the onset of the fever in the following manner:

The hair over the ventral aspect of the thorax was epilated with a concentrated solution of sodium sulfid and the denuded area disinfected with alcohol followed by tincture of iodine. With a needle and syringe, the chest wall was then punctured in the third or fourth intercostal space to the left of the sternum and blood aspirated directly from the heart. This was cultured in deep tubes of 2 percent glucose ascitic fluid agar in the manner previously described.

Table 1 shows the result of the blood cultures made upon this series of twenty-four guinea-pigs.

TABLE 1
BLOOD CULTURE STUDIES UPON ANIMALS

Number of Guinea-pig	Severity of Febrile Reaction	Day of Disease	Temperature When Culture Was Made F.	Quantity of Blood Cultured c.c.	Result in Colonies
12	Mild	1	104.4	5.0	0
13	Mild	2	104.6	5.0	0
16*	Mild	3	104.2	1.5	1
21	Severe	2	104.4	0.5	1
24	Severe	2	104.4	6.0	1
27	Severe	3	105.4	3.0	0
28	Severe	6	104.6	3.0	0
38	Mild	1	104.6	2.0	0
44	Mild	3	102.6	3.0	0
46	Mild	2	104.6	2.0	0
55	Mild	5	104.0	3.0	0
56	Severe	5	104.6	3.0	0
58	Severe	2	104.6	3.0	4
63	Mild	1	104.2	4.0	0
65	Severe	6†	102.8	1.0	4
69	Severe	2	104.8	1.5	1
74	Severe	2	104.6	4.5	11
75	Severe	2	104.4	3.0	0
76	Severe	6	105.2	2.0	0
85	Severe	7	105.6	2.5	0
87	Mild	2	104.6	2.0	0
102	Severe	2	104.6	3.0	0
103	Severe	3	106.2	5.0	1
105	Severe	4	105.8	5.0	0

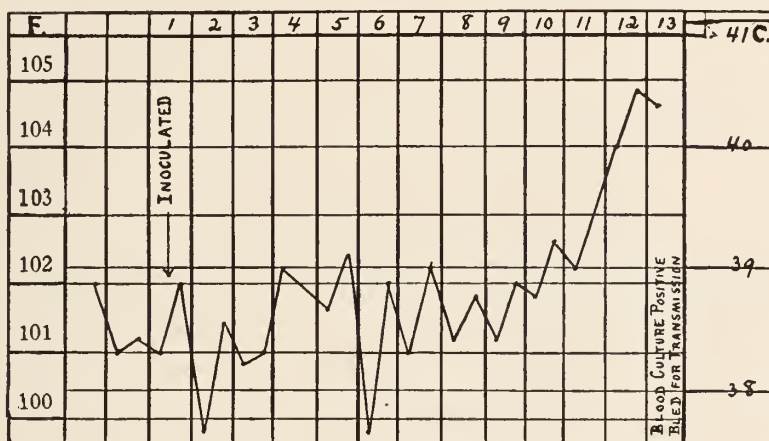
* Reaction in this animal followed inoculation with bacilli recovered from epidemic typhus Case 5. In all other animals in this series, the reaction was produced by inoculation of typhus blood.

† Twenty-four hours after crisis.

Of the twenty-four guinea-pigs in this series, in eight, or 33⅓ percent, a bacillus was isolated from the blood in pure culture which morphologically and culturally was identical with the one recovered from the blood of individuals with typhus fever. The fact that this percentage of positive blood cultures is lower than that obtained in the work on human cases, is undoubtedly for the most part due to the very small quantities of blood which we were forced to use for culture, the average amount being 3 c.c.

The febrile reactions at the time when the positive cultures were obtained were proved to be due to typhus fever. In four of these animals (Guinea-pigs 58, 65, 69, and 74) the proof was obtained by inoculating blood secured at the same time as the blood culture, into twelve other animals. All developed the disease after the usual incubation period, and, in some of these, immunity to re-inoculation was subsequently demonstrated. The blood of the others was found to be capable of reproducing the typical febrile reaction in normal guinea-pigs and not in those which had once had typhus fever. The results of the experiments on Guinea-pig 58 and on the three animals inoculated with its blood are presented as examples.

Guinea-Pig 58.—Inoculated intraperitoneally with 2.5 c.c. of defibrinated blood from Guinea-pig 50, diluted with an equal volume of normal salt solution. This was the third passage of the virus originally obtained from epidemic



Temperature Curve 1. Guinea-pig 58.

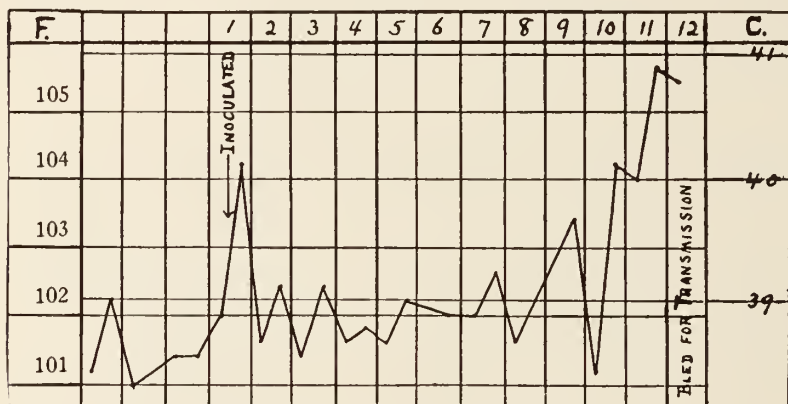
Reaction after inoculation with blood of Guinea-pig 50 (virus from epidemic typhus Case 10). Blood culture positive on second day of fever. Blood infectious for Guinea-pigs 27 and 69, but not for immune Guinea-pig 5.

typhus Case 10. The febrile reaction began suddenly on the twelfth day after inoculation. On the second day of the fever, 3 c.c. of blood were aspirated from the heart under aseptic precautions and cultured in two deep tubes of glucose serum agar. Five days later, three typical colonies appeared in one tube, and one in the other. The organisms from all four colonies were found to be both morphologically and culturally identical with the bacillus typhi-exanthematici.

Immediately after the blood culture was taken, 9 c.c. of blood were obtained from the animal's carotid artery; 3 c.c. of this blood, diluted with an equal vol-

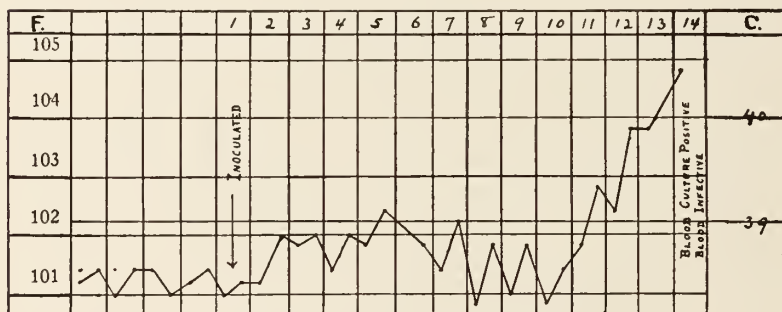
ume of salt solution, were injected intraperitoneally into two normal guinea-pigs, 27 and 29, and into an immune animal, Guinea-pig 5. Post mortem, no changes were discoverable in any organ except the spleen, which was enlarged, as a result of a hypertrophy of the Malpighian bodies.

Guinea-Pigs 27 and 69.—The temperature charts of Guinea-pigs 27 and 69 are supplied in place of detailed results. On the third day of the disease, both animals were bled from the carotid and their blood was found to be infectious for four normal guinea-pigs, but not infectious for a typhus-immune animal.



Temperature Curve 2. Guinea-pig 27.

Inoculated with blood from Guinea-pig 58. Blood infectious for other guinea-pigs.



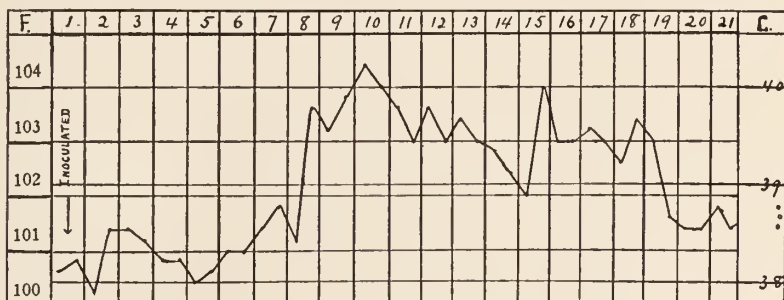
Temperature Curve 3. Guinea-pig 69.

Inoculated with blood from Guinea-pig 58. On the third day of reaction, blood culture positive and blood infectious for other guinea-pigs.

The postmortem findings were the same as in Guinea-pig 58. From 1.5 c.c. of blood obtained by cardiac puncture from Guinea-pig 69 before the transmission experiment, one colony of the bacillus typhi-exanthematici was grown.

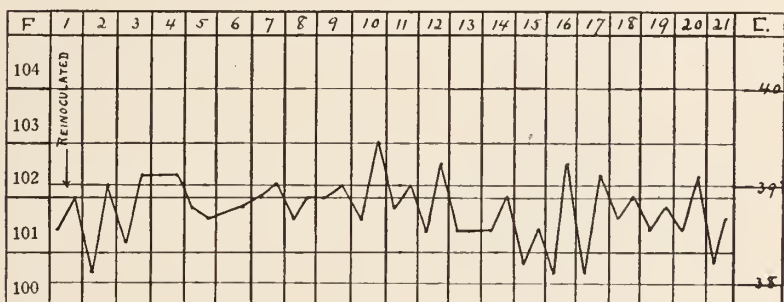
Guinea-Pig 5.—This animal had first been inoculated with 4 c.c. of defibrinated blood from epidemic typhus Case 4. It passed through a typical febrile

reaction (see temperature chart) and had completely recovered before it was used for this experiment. Two months after the first inoculation, it was re-inoculated with 3 c.c. of defibrinated blood from Guinea-pig 58, diluted with an equal volume of normal salt solution. Its temperature was observed twice daily for a period of thirty days thereafter and no febrile reaction occurred as in the control Guinea-pigs, 27 and 69.



Temperature Curve 4. Guinea-pig 5.

Reaction after inoculation with blood from epidemic typhus Case 4.

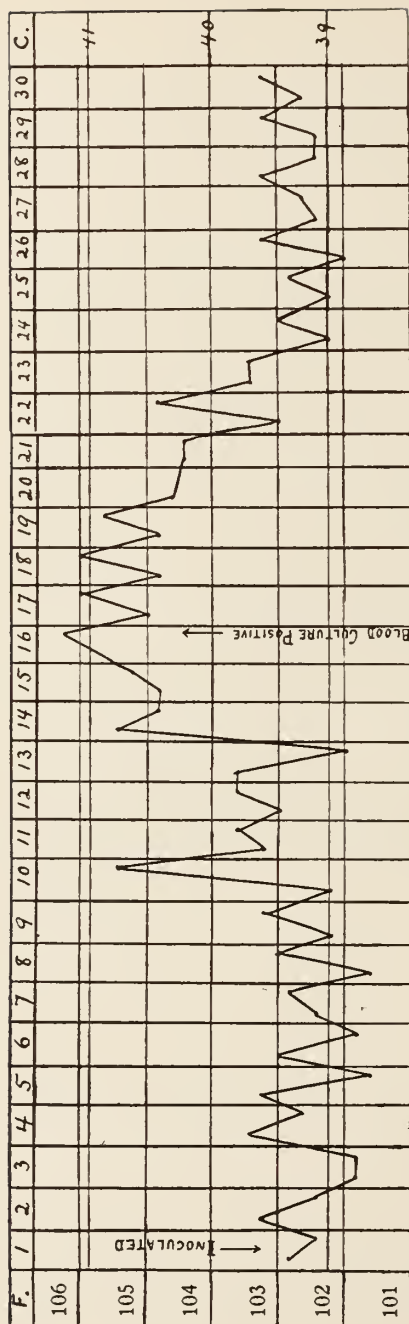


Temperature Curve 5. Guinea-pig 5.

Re-inoculated with blood from Guinea-pig 58. No reaction. (Controlled by Guinea-pigs 27 and 69.)

In Guinea-pig 103, in which the blood culture had been positive, the reaction was proved to be due to typhus fever by subsequently demonstrating that the animal was immune to a re-inoculation of another typhus virus.

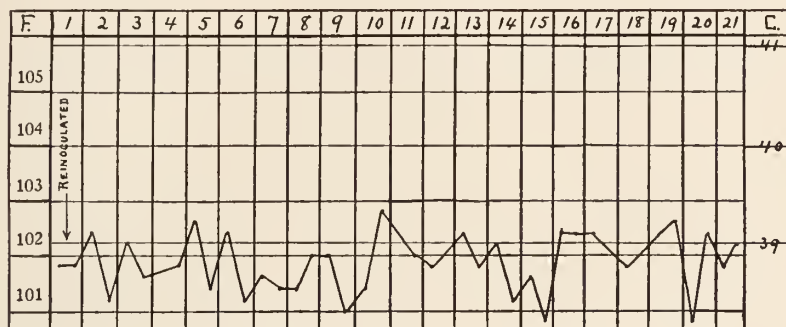
Guinea-Pig 103.—Inoculated intraperitoneally with 3 c.c. of defibrinated blood obtained from Guinea-Pig 63 at the height of the disease and diluted with an equal volume of normal salt solution. The virus had originally been obtained from epidemic typhus Case 10 and since that time had been transmitted through six guinea-pigs. Guinea-pig 103 developed the typical febrile reaction after an incubation period of fourteen days. On the third day of the disease, when



Temperature Curve 6. Guinea-pig 103.

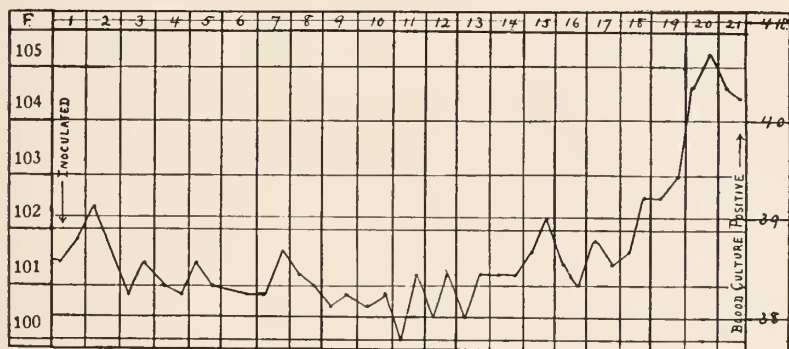
Inoculated with blood from Guinea-pig 63 (original virus obtained from epidemic typhus Case 10). Typical reaction. Blood culture positive on third day of fever.

the temperature was 106.2 F. (41.2 C.), a blood culture taken in the usual manner was positive. Five months after the termination of the illness, the animal was re-inoculated with 3.5 c.c. of defibrinated blood obtained from Guinea-pig 244 at the height of its disease and diluted with an equal volume of saline solution. This virus had been obtained by Anderson and Goldberger⁶ three and a half years previously from an endemic case in the wards of the Mount Sinai Hospital and since that time it had been transmitted through many generations of monkeys and guinea-pigs.



Temperature Curve 7. Guinea-pig 103.

Re-inoculated with blood from Guinea-pig 244 (endemic typhus Case W). No reaction, the previous illness having conferred immunity to typhus virus. Three control guinea-pigs developed typical febrile reactions.



Temperature Curve 8. Guinea-pig 24.

Inoculated with blood from epidemic typhus Case 10. Blood culture positive on second day of fever.

As seen in Temperature Curve 7, the animal proved resistant to this re-inoculation, altho three other control guinea-pigs, inoculated simultaneously with an equal amount of the blood, developed marked febrile reactions. The temperature observations were kept up for thirty-five days after the re-inoculation.

Of the other three animals with positive blood cultures, the reaction in Guinea-pig 16 was produced by the intraperitoneal injection of virulent bacilli. The reactions in Guinea-pigs 21 and 24 were produced by the injection of blood from epidemic typhus Case 10, which was also infectious for other guinea-pigs, producing in them the typical febrile reaction followed by immunity. As an example, see Temperature Curves 6 and 7 of Guinea-pig 103.

Therefore, in the eight animals with positive blood cultures, there can be no doubt that the febrile reactions were due to typhus fever.

Of the animals with negative blood cultures, Guinea-pig 13 was bled from the carotid immediately after the blood culture and this blood was inoculated into Guinea-pigs 61, 70, and 72 (3 c.c. into each). None of these animals developed the disease. Apparently, therefore, no viable bacilli were present in the blood of Guinea-pig 13 and this explains the negative result of the culture.

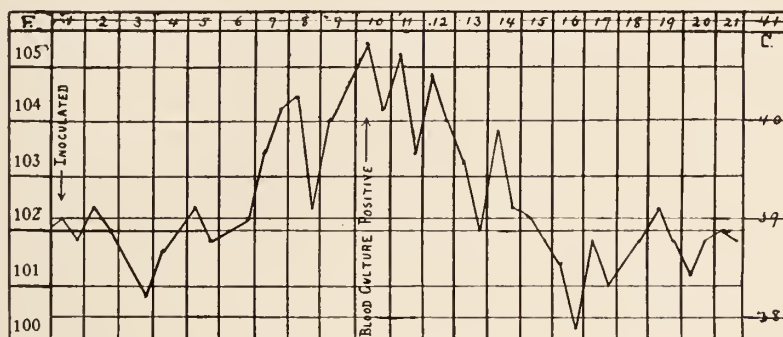
Blood from six other animals with negative blood cultures (Guinea-pigs 55, 56, 63, 75, 85, and 87) was inoculated into another series of thirteen guinea-pigs and all in this series developed the disease. This apparent discrepancy with the blood culture results possibly may have been due to the small quantity of blood used in the cultures. As will be shown later, in the inoculation experiments made with human blood, larger amounts were always available for culture and the blood culture results were therefore more accurate. In those experiments no such discrepancy between the blood culture results and the infectivity of the blood was ever noted.

In the positive blood cultures, the colonies averaged about one in a cubic centimeter of blood. This is more than was found in the patients with endemic typhus fever, but less than in individuals with epidemic typhus.

Blood cultures were also made on four monkeys in which the disease had been produced by inoculation with the blood of typhus guinea-pigs, 1-4 c.c. being used in each instance. In one monkey (B 1) the blood culture was positive.

Monkey B 1.—Inoculated intraperitoneally with 5 c.c. of defibrinated blood from Guinea-pig 247, diluted with an equal volume of normal salt solution. The original virus was the one obtained by Anderson and Goldberger⁶ three and a half years before from an endemic case. As a control, Guinea-pig 248 was also inoculated with 3 c.c. of the blood of Guinea-pig 247 and it developed the disease after a nine-day incubation period. In Monkey B 1, the disease began suddenly on the eighth day of the experiment.

On the third day of the fever, when the temperature reached 105.4 F. (40.8 C.), 5.5 c.c. of blood were aspirated from a vein in the leg under aseptic precautions. Two cubic centimeters of this were cultured in two deep tubes of glucose serum agar, and in one of the tubes one colony appeared five days later. The organisms from this colony proved to be morphologically and culturally identical with the bacillus typhi-exanthematici. Three and one-half cubic centimeters of the blood were inoculated intraperitoneally into Guinea-pig 256. After an incubation period of sixteen days, it developed a characteristic febrile reaction which lasted six days.



Temperature Curve 9. Monkey B 1.

Inoculated with blood from Guinea-pig 247. Original virus had been obtained from a patient with endemic typhus fever three and a half years before. Blood culture positive on fourth day of fever.

This experiment is of importance because it demonstrates that in monkeys with typhus fever the same bacillus can also be isolated from the blood. Of especial interest is the fact that the virus with which this monkey was infected was the one obtained by Anderson and Goldberger⁶ from a patient with endemic typhus fever three and a half years before.

RELATION OF BLOOD CULTURES TO THE SEVERITY OF THE DISEASE

Of nine guinea-pigs with mild febrile reactions, in only one was the blood culture positive. On the other hand, in fifteen animals with severe reactions, the blood culture was positive in seven, or almost 50 percent. This relation between the blood cultures and the severity of the disease was also observed in the human typhus cases (q. v.). It is undoubtedly very suggestive as to the causal relationship of the bacterium to the disease.

TABLE 2
RELATION OF BLOOD CULTURES TO THE STAGE OF THE DISEASE

Day of Culture	Number of Animals Cultured	Number of Positive Blood Cultures
1st day	3	0
2nd day	10	5
3rd day	4	2
4th to 7th day	7	1

As the table indicates, blood cultures taken on the second and third day of the disease (that is 24-72 hours after the onset) were positive in 50 percent of the animals.

In this series of experiments also, the blood cultures were purposely taken at different stages of the disease, as outlined in Table 2.

This observation assumes especial interest when the temperature curves of thirty-five other animals, which were permitted to live for the entire course of their disease, are analyzed. Of this series, the highest temperature was reached during the first twenty-four hours in two animals, on the second day in twelve, on the third day in eleven, on the fourth day in seven, on the fifth day in two, and on the sixth day in one animal.

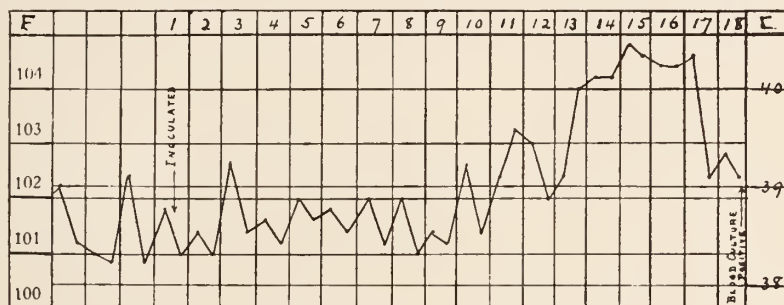
In the majority of instances (66 percent), therefore, the fever reached its highest point on the second or third day of the disease, in other words, 24-72 hours after the onset. (Anderson² reports that, in his guinea-pig experiments, the highest temperature was usually reached 36-72 hours after the onset.) This is the period when in the infected animals the blood culture is most frequently positive. It is still another demonstration of the relation between the severity of the disease and the numerical frequency of the organisms in the circulating blood.

POSITIVE BLOOD CULTURES AFTER THE CRISIS

Guinea-pig 65 of the blood culture series requires special mention because of the fact that the positive blood culture was obtained 24-36 hours after the crisis.

Guinea-Pig 65.—Inoculated with 3 c.c. of defibrinated blood obtained from Guinea-pig 68 on the third day of the disease. The blood was diluted with an equal volume of normal salt solution before injection. The original virus had been obtained from epidemic typhus Case 10 and since that time had been transmitted through four successive guinea-pigs. The fever began suddenly on the fourteenth day of the experiment and lasted five days.

A blood culture was taken 24-36 hours after the crisis, when the temperature was normal. From 1 c.c. of blood used for the culture, four colonies were grown, a proportionately larger number than in any of the blood cultures in the other animals of the series. When 3.5 c.c. of blood, obtained at the same time as the blood for culture, were injected intraperitoneally into Guinea-pig 98, it developed a typical reaction after an incubation period of seven days.



Temperature Curve 10. Guinea-pig 65.

Reaction after inoculation with blood from Guinea-pig 68. Crisis on fifth day of fever. Blood culture positive 24-36 hours after crisis.

The finding of viable bacteria in the blood of an animal 24-36 hours after the crisis is in accord with the observations recorded by Dr. Plotz in his paper of the presence of bacilli in the blood of two typhus patients, 12 and 36 hours after the crisis. Nicolle and Conseil⁹⁸ believed the blood to be infective 36-48 hours after the crisis, but their observations are not convincing. Anderson and Goldberger¹⁶ have found the blood to be infective for animals 12 and 32 hours after the crisis. In our animal, not only was the blood culture positive 24-36 hours after the crisis, but 3.5 c.c. of blood obtained at the same time were infective for Guinea-pig 98. After an incubation period of seven days, Guinea-pig 98 ran a typical febrile course, reaching as high as 105.2 F. (40.7 C.) and lasting six days.

The observation of the presence of bacilli in the blood 24-36 hours after the crisis, and the finding that the blood is still infective during this time, are important for several reasons. First, it completely eliminates the possibility that the crisis in typhus fever may be due to a sudden destruction of the bacteria. The critical ending of the disease is therefore most probably due to the final consummation of a cellular immunity on the part of the body to the bacilli. In some cases, at least, it is only later with the subsequent development of bacterial antibodies during the 24-48 hours after the crisis that the bacilli themselves are eventually killed off. (During the precritical stage of the disease,

probably the chief factors tending to limit the multiplication of the bacilli are opsonization and phagocytosis, as has been suggested in the preceding section of this paper.) This explanation of the mechanism of the crisis in typhus fever also received substantial confirmation in the serologic studies of Dr. Olitsky. He was actually able to demonstrate that, in many instances in men and monkeys, the specific bacterial antibodies (agglutinins, precipitins, complement-fixation bodies, etc.) only begin to appear in the blood during the first few days after the crisis.

The demonstration of the presence of bacilli in the blood and its infectivity for so long a time after the crisis is also of interest from another standpoint. It indicates that, during this early period of convalescence, the patient may be a bacillus carrier and hence may still be a source from which infection can spread.

PATHOGENICITY OF THE BACILLI FROM EPIDEMIC TYPHUS CASES

Two strains of bacilli obtained from two of the epidemic typhus cases (epidemic typhus Cases 1 and 5) were inoculated intraperitoneally into guinea-pigs. In each instance the material used for the inoculation consisted of a subculture of the organism, which had grown on a slant of 0.5 percent glucose ascitic fluid agar in a Buchner tube for three days. The growth was scraped from the surface of the slant with a platinum loop and emulsified in 2 c.c. of normal salt solution. This represented the inoculated dose.

In epidemic typhus Case 1, the colonies did not appear in the blood culture tubes until the fourteenth day, so that including the time subsequently consumed in the identification and study of the organism, the bacilli were out of the body for thirty-three days before they were inoculated into Guinea-pig 8. The organism recovered from epidemic typhus Case 5 was out of the body sixteen days before it was inoculated into Guinea-pig 16. (Guinea-pigs 8 and 16 were isolated in individual cages which were separated from one another and from all other animals by wooden partitions.)

During the first twenty-four hours after the inoculation, the animals developed a slight, transient elevation in temperature, the significance of which will be discussed later. The temperature of Guinea-pig 8 then remained normal, that is, below 102 F. (38.8 C.) until the sixth day after inoculation, when a sudden rise to 104 F. (40 C.) occurred. The animal then ran a febrile course similar to that of animals after inoculation with the blood of typhus patients. The temperature

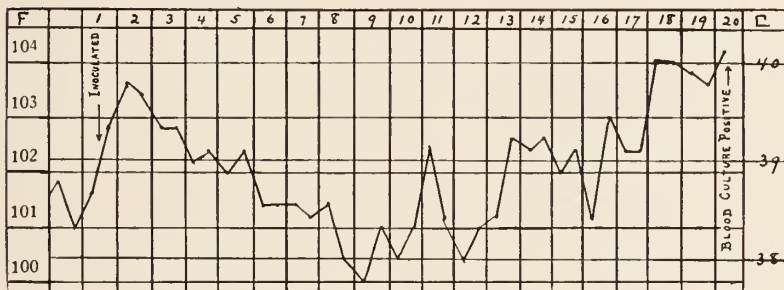
remained about 104 F. (40 C.) for four days and then gradually fell to normal, reaching 102 F. (38.8 C.) two and a half days later. During the next five days the temperature remained normal, that is, below 102.4 F. (39 C.).

In order to test its immunity, the animal was then re-inoculated with 3 c.c. of defibrinated blood obtained from epidemic typhus Case 10, and died several hours later as a direct result of the inoculation.



Temperature Curve 11. Guinea-pig 8.

Reaction after inoculation with bacteria (strain from epidemic typhus Case 1).



Temperature Curve 12. Guinea-pig 16.

Reaction after inoculation with bacteria (strain from epidemic typhus Case 5). Bacteria recovered from blood during reaction.

Unfortunately this was very early in our work when we had not yet learned to delay re-inoculation until the animal had recovered its full weight and strength.

The result obtained with Guinea-pig 16 was still more striking. After an incubation period of eighteen days, it developed a sudden rise of temperature to 104 F. (40 C.). The fever continued at about this level, and on the third day after the onset a blood culture was made.

After epilation and disinfection of the skin over the ventral aspect of the thorax in the usual manner, 1.5 c.c. of blood were aspirated from the heart and cultured in two deep tubes of 2 percent glucose ascitic fluid agar. The animal died shortly after the operation of intrapericardiac hemorrhage.

At autopsy, no anatomic or histologic lesions were found in any organ except the spleen. The latter was enlarged to two or three times its normal size; the Malpighian bodies were markedly hypertrophied so that they were easily visible macroscopically. These are the lesions which we have always found in guinea-pigs which have been killed, or have died, during the course of a typhus fever. Ricketts and Wilder have regarded the absence of any pathologic lesion in any of the viscera as characteristic of typhus fever in the monkey. But in our numerous experiments with the guinea-pig, altho all other viscera remained microscopically, as well as macroscopically normal, we have never failed to find a marked enlargement of the spleen, due chiefly to a hypertrophy of the Malpighian bodies.

Seven days after the blood culture was made, a characteristic colony appeared in one of the culture tubes. This organism upon sub-culture proved to be both morphologically and culturally identical with *Bacillus typhi-exanthematici*.

These experiments prove that bacilli recently isolated from epidemic cases of typhus fever are pathogenic for guinea-pigs. They further demonstrate that with such organisms a disease can be produced in these animals which in its incubation period and its febrile course is similar to typhus fever as reproduced in guinea-pigs by the inoculation of typhus blood. And finally, what is perhaps most important, they demonstrate that during the febrile period of the disease an organism identical with that which was inoculated can be recovered from the circulating blood.

LOSS OF VIRULENCE AFTER ISOLATION

Other guinea-pigs and monkeys were subsequently inoculated with the two strains of bacilli used in the experiments just described (epidemic typhus Cases 1 and 5) after the strains had been retransplanted on slants of 2 percent glucose ascitic fluid agar a number of times; both strains were then found to have completely lost their virulence. Three other strains of organisms, from epidemic typhus Cases 2, 4, and 6, after they had been on artificial media for more than three or four

weeks, were also inoculated into guinea-pigs and monkeys, with negative results. In each instance the inoculation was followed only by a slight, transitory rise in temperature during the first twenty-four hours, a reaction also observed in the experiments with virulent organisms.

The regular occurrence of this rise in temperature during the first twenty-four hours after inoculation of epidemic typhus organisms we believe to be due to the absorption of bacterial endotoxins. If, before the inoculation, the organisms were suspended for one-half to one hour in a 1:20 to 1:100 dilution of typhus immune serum, that is, serum from an individual convalescent from typhus fever, no such rise in temperature occurred. This was considered as evidence of a neutralization of the bacterial toxin by an antitoxin in the immune serum. After cultivation on artificial media for more than a month, all strains of epidemic typhus organisms also lost this toxic action.

In spite of repeated attempts by passages through mice and guinea-pigs, it was found impossible to restore the virulence of any of the strains after they had once lost it. Other attempts were made by growing the organism in citrated blood and in magnesium carbonate broth, but these were unsuccessful.

This rapid loss of virulence explains why the reproduction of typhus fever in animals by inoculation of the bacilli was not done more extensively. The positive blood cultures in cases of epidemic typhus fever were obtained at the very beginning of the work. Subsequently, when the importance of inoculating the organisms into animals within the shortest possible time after isolation was realized, no more cases of epidemic typhus fever were available.

We hope to have the opportunity very shortly to amplify this phase of the work. It is important, however, that we have demonstrated that with bacilli recently isolated from epidemic cases of typhus fever the disease can be reproduced in animals, and that at the height of disease so produced the identical organism can again be recovered from the blood.

VIRULENCE OF BACILLI FROM ENDEMIC CASES

Experiments carried out with six strains of bacilli from the endemic typhus cases (Cases 1, 2, 18, 23, 27, and 40) and with those isolated from three animals (Guinea-pigs 21, 24, and 103) with typhus fever, revealed the fact that they lost their virulence outside the body still more rapidly than did the epidemic typhus strains. Before suffi-

cient growth could be obtained for inoculation, the organisms were on artificial media for two to three weeks, or even longer. When finally ready for inoculation, such organisms were not only avirulent, but they did not even possess the power of producing a toxic rise in temperature after inoculation, a property only lost by the epidemic typhus organisms after cultivation for over a month.

Both morphologically and culturally the organisms isolated from the epidemic and the endemic types of the disease proved to be identical, and both were found to possess the same specific antigenic properties. This variation in virulence and in toxin production is the only essential difference which we have been able to demonstrate between the organisms derived from the two sources. Its importance lies in that it supplies us with a possible reason for the difference in the degree of bacteriemia and in the severity of the two diseases.

RELATION BETWEEN THE NUMBER OF BACILLI IN TYPHUS BLOOD AND ITS INFECTIVITY

The observations presented thus far in this section of our report have demonstrated the association of the bacillus recovered from cases of typhus fever with the disease as reproduced in the experimental animals. Further evidence from the experimental standpoint that this organism is the etiologic agent in typhus fever was readily obtained by a study of the relation between the number of the bacilli in typhus blood and its infectivity.

In this series of experiments, eight monkeys and forty-three guinea-pigs were inoculated with blood obtained from twenty-three endemic cases of typhus fever and five epidemic cases. The blood for inoculation was obtained at the same time as the blood for culture and frequently with the same syringe. After defibrination, 2-9 c.c., diluted with an equal volume of normal salt solution, were injected intraperitoneally into each animal. The blood from the endemic cases was, as a rule, injected within half an hour after it was obtained. The blood from the epidemic cases had to be carried from the quarantine station on Swinburne Island, New York, and was sometimes out of the body as long as three or four hours.

In Table 3, Column 5, is listed the average number of organisms apparently contained in the quantity of blood inoculated into each animal. This was estimated from the total number of bacterial colonies which developed in the blood culture tubes. It is quite pos-

sible that some organisms may have lodged in the upper 3-5 cm. of the column of medium and may not have multiplied because of the incomplete anaerobiosis. Also, as soon as a tube is broken open and a subculture of a colony made, those colonies which may have appeared subsequently are lost. Upon analysis, however, the latter source of error in the statistics is found to be relatively slight. For altho the time when the colonies became visible in the different blood cultures varied from three to sixteen days, in each individual culture all the

TABLE 3

RELATION OF NUMBER OF ORGANISMS (AS DETERMINED BY BLOOD CULTURE) TO INFECTIVITY OF BLOOD

Case	Amount of Blood Used for Culture in c.c.	Colonies of Organisms	Amount of Blood Used for Inoculation in Each Animal in c.c.	Average Number of Organisms in the Amount of Blood Injected	Number of Animals Inoculated*	Blood Infective
Endemic 8.....	14	0	3.0	0	3	0
Endemic 11.....	14	0	7.5	0	2	0
Endemic 12.....	12	0	9	0	2	0
Endemic 14.....	16	0	5	0	2	0
Endemic 15.....	12	0	3	0	1	0
Endemic 17.....	20	0	3	0	1	0
Endemic 19.....	12	0	5	0	2	0
Endemic 25.....	14	0	2.5, 2.5, 5	0	3	0
Endemic 26.....	16	0	3	0	1	0
Endemic 27.....	16	0	3	0	1	0
Endemic 28.....	16	0	3	0	4	0
Endemic 30.....	15	0	5	0	1	0
Endemic 31.....	15	0	3 and 5	0	2	0
Endemic 37.....	12	0	3	0	2	0
Endemic 18.....	16	1	4	>1	1	0
Endemic 23.....	15	1	3	>1	3	0
Endemic 32.....	15	1	3	>1	1	0
Endemic 34.....	10	1	3	>1	1	0
Endemic 36.....	16	2	2	>1	2	0
Endemic 9.....	4	1	5	>1	3	0
Endemic 39.....	14	4	3 and 4.5	>1	2	0
Endemic 20.....	14	3	6	>1	2	0
Epidemic 1.....	6	2	5	>2	1	0
Epidemic 2.....	6	3	4, 4 and 5	2	3	+
Epidemic 40.....	15	9	3 and 4	2	2	+
Epidemic 6.....	6	9	5	8+	1	+
Epidemic 4.....	6	14	4	9	1	+
Epidemic 5.....	6	28	5	23	1	+

* One monkey was used in each of the series of experiments made with blood from endemic typhus Cases 25, 28, 30, 31, 39, 40, and epidemic typhus Cases 1 and 2. All the other animals listed in this column were guinea-pigs.

colonies usually appeared in the tubes within twenty-four to forty-eight hours. Tubes were seldom opened until seventy-two hours after the first colonies appeared.

In fourteen of the twenty-eight cases in this series, the blood cultures were negative, altho large amounts of blood were used, 12-20 c.c. Blood from these patients, in quantities ranging from 2.5 to 9.0 c.c.,

was likewise inoculated into twenty-seven animals, four monkeys and twenty-three guinea-pigs. None of the animals developed a febrile reaction. Nor was immunity ever produced, for all the animals reacted in the typical manner to a subsequent re-inoculation of active typhus blood.

In the other fourteen cases, the blood cultures were positive, the number of colonies developing in the different cultures ranging from one to twenty-eight. In nine of these fourteen patients (endemic typhus Cases 9, 18, 20, 23, 32, 34, 36, 39, and epidemic typhus Case 1), the blood cultures contained relatively few colonies, so that the estimated number of bacilli in the quantities of blood injected averaged less than two. Sixteen animals, two monkeys and fourteen guinea-pigs, were inoculated with blood from these patients. In no instance did the inoculation result in a febrile reaction or in an immunity.

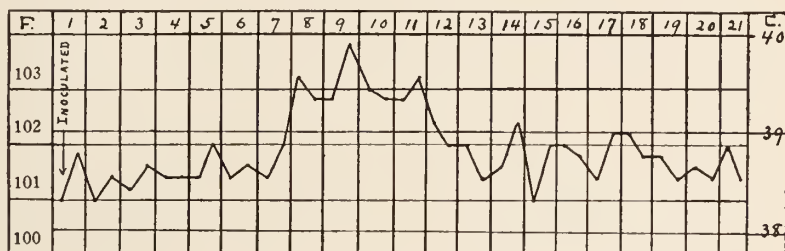
In the remaining five patients (epidemic typhus Cases 2, 4, 5, 6, and endemic typhus Case 40), the blood cultures showed the organisms to be more numerous. The average number of bacilli estimated as present in the quantities of blood used for the animal inoculations ranged from two to twenty-three. The blood from all five cases was found to be infective for animals. After the characteristic incubation period, the animals ran a typical febrile course.

The experiments carried out with the blood of epidemic typhus Case 2 is especially interesting. Of the five typhus patients whose blood was infective, the blood from this patient apparently contained the fewest organisms. It contained proportionately slightly more organisms, however, than epidemic typhus Case 1, or endemic typhus Case 20, the blood from which was not infective. Into each of two guinea-pigs (Guinea-pigs 2 and 3) 4 c.c. of blood were inoculated and 5 c.c. into a monkey (Monkey A 2). The monkey did not react. Both guinea-pigs developed exceedingly mild, tho quite definite, febrile reactions.

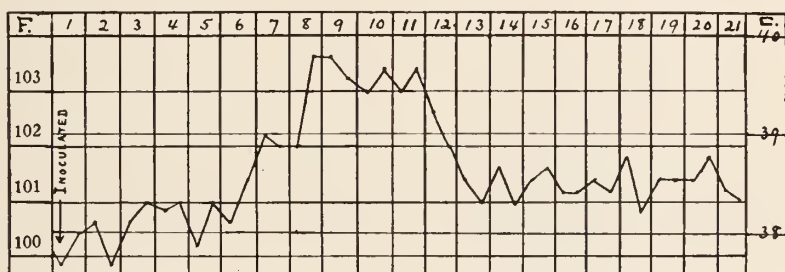
Guinea-Pig 2.—After the inoculation with blood from epidemic typhus Case 2, the temperature remained normal until the seventh day, ranging between 101 F. (38.4 C.) and 102 F. (38.8 C.). On the seventh day after inoculation, it rose abruptly to 103.2 F. (39.5 C.) and remained about this level for five days. The highest temperature 103.8 F. (39.9 C.) was reached on the third day after the onset. On the fifth day the temperature fell to normal, 102 F (38.8 C.), and remained so for eight days, when the animal died immediately after a re-inoculation with typhus blood.

Guinea-Pig 3.—Temperature normal, about 101 F. (38.4 C.), until the sixth day after inoculation, when it rose to 102.2 F. (39 C.). Eight days after inoculation, it rose suddenly to 103.6 F. (39.8 C.) and remained high for four days. After the defervescence of the fever by rapid lysis, the temperature remained below 101.8 F. (38.8 C.) for three weeks.

Altho neither animal was tested for its immunity, the febrile reactions in both were undoubtedly due to typhus fever. They occurred in both guinea-pigs after the characteristic incubation period, seven and eight days respectively, and ran a typical course.



Temperature Curve 13. Guinea-pig 2.
Reaction after inoculation with blood from epidemic typhus Case 2.

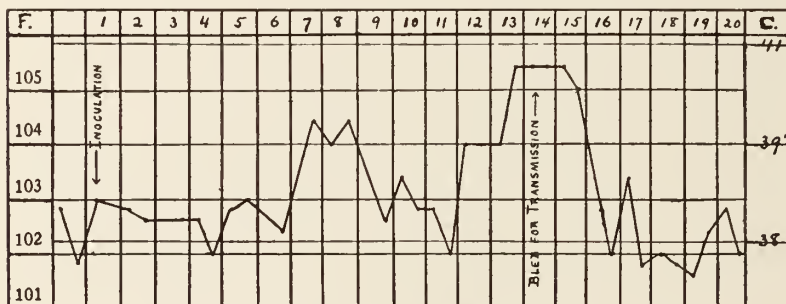


Temperature Curve 14. Guinea-pig 3.
Reaction after inoculation with blood from epidemic typhus Case 2.

The fact that the monkey did not react does not necessarily indicate that it is less susceptible to the disease than the guinea-pig. For it is not improbable that the 5 c.c. of blood injected into the monkey may perchance have contained fewer organisms than the 4 c.c. injected into the guinea-pigs.

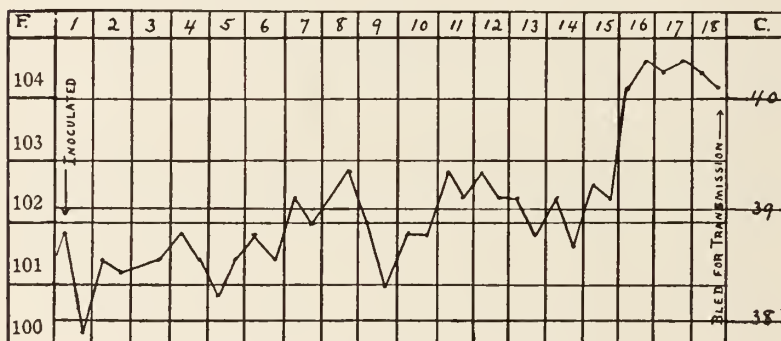
The following reports of the two experiments carried out with the blood of endemic typhus Case 40 are supplied as additional examples of temperature reactions in this series.

Monkey B 7.—Inoculated intraperitoneally with 4 c.c. of blood obtained from endemic typhus Case 40 five days before the crisis. The blood was defibrinated and diluted with an equal volume of normal salt solution before injection. Following the inoculation, the temperature remained normal until the ninth day of the experiment. On the fifteenth day of the experiment, when the temperature was 105.4 F. (40.8 C.), the animal was bled, 5.5 c.c. of blood being obtained. Two and a half cubic centimeters injected into Guinea-pig 229 resulted in a febrile reaction after an incubation period of sixteen days, and the blood during this period was found to be infectious for three other guinea-pigs. Three cubic centimeters injected into Monkey B 5 failed to produce a febrile reaction.



Temperature Curve 15 Monkey B 7.

Reaction after inoculation with blood from endemic typhus Case 40.



Temperature Curve 16. Guinea-pig 277.

Reaction after inoculation with blood from endemic typhus Case 40.

Guinea-Pig 277.—Inoculated intraperitoneally with 3 c.c. of blood obtained from endemic typhus Case 40, defibrinated and diluted as in experiment on Monkey B 7. On the sixteenth day of the experiment, the temperature rose suddenly to 104 F. (40 C.).

On the fourth day after the onset of the fever, the animal was bled to death from the carotid. Post mortem, the spleen was found to be almost three times the normal size and the Malpighian bodies were very prominent. All other viscera appeared normal.

With the blood of Guinea-pig 277, the disease was reproduced in three other animals and eventually transmitted through nine generations of guinea-pigs. Some of these animals were subsequently found to be resistant to an inoculation of another typhus virus. In others, the blood during the febrile period was found to be infectious for normal guinea-pigs but not for animals which had once had the disease.

Because of the possibility that all the viable organisms in the blood may not have developed into colonies on culture, we can not commit ourselves to an expression of opinion as to the degree of accuracy of the statistics in Columns 3 and 5 of Table 3. Nor is it possible to ascertain just how many bacilli constitute the minimal infecting dose. But even if we merely view the results of these experiments most conservatively as comparative observations, they are still of great significance.

The uniformity of the twenty-eight observations tabulated in Table 3 clearly indicates that typhus blood which contains no bacilli, or only very few bacilli, is *not* infective for animals. Typhus blood in which the bacilli are more numerous is infective. These experiments therefore demonstrate that infectivity is absolutely dependent upon the presence of a sufficient number of these bacilli. This in itself is conclusive evidence of their etiologic significance.

SIGNIFICANCE OF RESULTS OF THE FOREGOING OBSERVATIONS FOR THE PROBLEM OF LOUSE TRANSMISSION

The question naturally arises as to the significance of the results of the bacteriologic and experimental observations for the problem of louse transmission. We ourselves have not been able to carry on any experiments with lice. But at least two of the observations made in the preceding studies—the fact that the organism is a bacillus and that it occurs in the blood in typhus fever only in relatively small numbers—have considerable bearing upon some of the still obscure phenomena of louse transmission.

The presence of so few bacilli in the blood must render it relatively difficult for lice to become infected. That this is true is evidenced by the great difficulty which previous workers have experienced in transferring an infection experimentally by means of lice. If the experiments of previous workers^{16, 92, 98, 131} are analyzed, the striking feature is seen to be the fact that large numbers of lice, often more than one hundred, were necessary in order to transfer the disease. These lice were usually fed upon infected individuals a number of

times before they were used for the experiments. Even under these conditions, usually no fever or other manifestations of the disease were produced, the only proof that a transfer of virus had occurred being the subsequent demonstration of an immunity to re-infection.

Thirty-seven lice fed on an infected individual on two successive days is the minimal number with which it has thus far been possible to reproduce experimentally the typical febrile reaction to the disease. And in this experiment, performed by Nicolle, Blanc, and Conseil,⁹² the entire abdominal contents of the thirty-seven lice were injected intraperitoneally into a monkey.

In view of the paucity of the bacilli in the blood of infected individuals, we are forced to the conclusion that in all the experiments repeated feedings of a very large number of lice were necessary before sufficient blood had passed through them for at least a few to become infected.

Another observation which has been especially puzzling to all investigators^{16, 92, 98, 181} has been the fact that the lice seem only to become infective five to six or even ten days after the first feeding. Nicolle⁹⁸ believed that this was evidence that the micro-organism was a protozoon, and that this length of time was needed for it to pass through some developmental cycle in the body of the louse, preliminary to becoming infective. Since the micro-organism is a bacillus, the only possible explanation is the one suggested originally by Ricketts and Wilder,¹³¹ that during this period the bacilli in the lice are undergoing an increase in numbers or in virulence.

There can be no doubt that the virulence is at least maintained in the body of the louse. An increase in the virulence of the bacilli during their residence in the louse is not improbable, but there is as yet no positive proof that it occurs. On the other hand, because of the relative infrequency of the organisms in the blood of infected individuals, even large numbers of lice can obtain only very few bacilli during the few feedings that they are given in the experiments. Undoubtedly, some days must therefore transpire before the bacilli in the infected lice have increased sufficiently in numbers to become potent. Our observations, therefore, supply an explanation for some of the previously inexplicable features of the louse experiments. It must be remembered however that under normal conditions the lice which ordinarily swarm over an infected individual have the opportunity to feed much more frequently and for a much longer time.

The amount of blood which therefore passes through each individual louse during the entire course of a disease is much greater than in the experiments. Consequently, by the time the disease has run its course, very few lice, or even a single louse, probably is often capable of transmitting the infection.

From our observations we are able to throw some light upon another important phase of louse transmission. Nicolle, Conor, and Conseil,⁹⁴ Ricketts and Wilder,¹¹⁸ and Anderson and Goldberger¹⁶ have noted that monkeys which have been exposed to the bites of infected lice may subsequently develop an immunity without having had fever or any other sign of illness. The two observations to be described, which we have recently been able to make on two individuals after exposure to the infection of typhus fever, demonstrate that the same thing may occur in human beings.

Dr. B.—During the course of this work it had been customary for one of us (Dr. B.) to bleed animals for the purposes of study, virus transmission, etc. No special precautions were taken to avoid infection, the blood of the infected animals often remaining on his hands throughout a series of experiments lasting over an hour. About five months ago, Dr. B. experienced some slight malaise and general muscular pains for several days, but they were not marked enough to require any attention.

Three or four weeks later his blood was studied serologically, preliminary to the proposed administration of vaccine. Much to our surprise, his serum gave a + + + + complement fixation with typhus antigen and agglutinated a stock of typhus agglutinin in a dilution of 1:500 (see serologic studies). As we had only observed serologic reactions of this degree in individuals who were convalescing from typhus fever, we had reason for believing that Dr. B. had recently passed through an infection, altho he had shown only exceedingly slight clinical manifestations of illness. This was confirmed by the fact that, as in convalescents from typhus fever, the serologic phenomena gradually diminished in intensity after the first month, so that by the end of four months both complement fixation and agglutination became practically negative.

Whether the infection occurred with or without the intermediary agency of the louse could not be ascertained. Dr. B. came into contact with most of the patients with endemic typhus fever, but usually only after their clothing had been removed and they had been bathed.

Miss L.—With the observation on Dr. B. in mind, studies were also made on two nurses, Miss L. and Miss H., who had recently returned from Serbia after nursing for almost three months at Valjevo. The patients under their care had been covered with vermin. Miss L. asserted that she had been repeatedly bitten by lice and that on at least two occasions she had removed lice from her clothes. The serologic studies on Miss H. were negative. The serum of Miss L. gave a + + + + fixation with typhus antigen and firmly agglutinated typhus bacilli in a dilution of 1:200.

These observations permit of only one interpretation. Both Dr. B. and Miss L. were exposed to infection and shortly thereafter their blood was found to contain specific antibodies in amounts only observed in individuals who have recently recovered from the disease. It is therefore reasonable to assume that at the time of their exposure they had been infected with the bacilli, but in quantities insufficient to induce the clinical manifestations of the disease. In view of the absolute immunity of monkeys after exposure to a similar infection,^{16, 94, 118} the high titer of the specific bacterial antibodies in the blood of Dr. B. and Miss L. can be considered as evidence of at least a partial, if not an absolute, immunity.

As we have met with two such instances in our small experience, they are probably not very uncommon.* In epidemics, such individuals who have become immune without actually having had the disease may subsequently act as carriers of the infected lice.

CONCLUSIONS

In the experiments cited in the preceding pages it has been shown that a bacillus, identical with that recovered from patients with typhus fever, can also be isolated from the blood of animals in which the disease has been reproduced by inoculation of typhus blood. In such animals, the frequency of the bacilli in the blood is directly proportionate to the severity of the illness. In individual animals it is greatest at the height of the disease.

It has also been shown that with the bacilli isolated from epidemic cases of typhus fever, it is possible to reproduce the disease in animals. And furthermore, at the height of the disease in such animals, the identical organism can be recovered from the circulating blood.

Finally, it has been shown that typhus blood is only infective if it contains a sufficient number of these bacilli.

* Since this was written, we have had the opportunity of studying eight other contacts, individuals who were exposed to the infection of typhus fever but did not develop the disease. The serum of three of the contacts gave a ++ to +++ fixation with typhus antigen and agglutinated the bacilli in dilutions from 1:200 to 1:500. Therefore, specific agglutinins and complement-fixing bodies have been demonstrable in the blood of five out of the ten contacts studied. This is very significant in view of the fact that in the sera of over one hundred non-contact controls, complement fixation was never observed and specific agglutinins were never found in dilutions above 1:50. The studies on typhus contacts will be continued and will be reported later. It is already apparent however that individuals who are exposed to typhus fever may react with the production of specific antibodies without having had any clinical evidences of the disease.

Aside from its importance for the problem of the epidemiology of the disease, this fact may be of significance as regards vaccine therapy. Should non-sensitized or sensitized vaccines prepared from epidemic and endemic strains of the typhus bacillus prove unsatisfactory, this observation suggests the advisability of testing, with all proper precautions, the value of the inoculation of small numbers of living bacilli beginning with those obtained from endemic cases.

From these observations and the results of the bacteriologic and serologic studies, we believe ourselves justified in concluding that this bacterium is the causative agent in typhus exanthematicus.

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EXPLANATION OF PLATE 1

Fig. 1.—*Bacillus typhi-exanthematici*: Gram's stain. $\times 1000$.

Fig. 2.—Colony of *Bacillus typhi-exanthematici* — with area of precipitation — in original blood culture.

Fig. 3.—Tube showing growth (seven days) of *Bacillus typhi-exanthematici* on serum glucose agar. Note whitening of medium (precipitation).

Fig. 4.—A control tube of glucose serum agar.



Figure 1



Figure 2



Figure 3



Figure 4

ON AN EPIDEMIC OF SORE THROAT AND THE VIRULENCE OF STREPTOCOCCI ISOLATED FROM THE MILK *

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The occurrence of virulent streptococci in milk and the relation of infected milk to epidemics of "septic sore throat" are now generally recognized. The object of this paper is not so much to report an epidemic of this character, which was recognized as such from a study of the cultural and other characteristics of the streptococci isolated from the patients' throats and sputum, as to detail the peculiar pathogenic powers of the streptococci from the infected milk.

The epidemic occurred during February, 1914, in Elmhurst, Illinois, a city of 3,800 people. The infections, as compared with the usual epidemics of sore throat, were less severe, and the organisms isolated were of a moderate grade of virulence, being distinctly less virulent than the strains of streptococci isolated from the throats in cases of the milk-borne epidemic of sore throat occurring in Chicago in the winter of 1911-12, and studied by Capps and Miller,¹ Davis,² and Rosenow.³ In some cases, the initial infection of "sore throat" was followed by bronchopneumonia, and attacks of "rheumatic" myositis and arthritis. Investigation showed that some thirty cases had occurred in families using milk from one dairy. Dr. Langhorst, of the board of health in Elmhurst, co-operated with us in securing cultures from the cases, and the owners of the dairy supplied us with samples of milk from the various farms, eight in all.

The streptococci isolated from the throats produced on blood agar plates rather large, moist colonies, surrounded by narrow, ill-defined, hazy zones of hemolysis, corresponding closely in these characteristics with the organisms isolated from the milk, and indistinguishable from the milk organisms after animal passage. The method of isolating the organisms from the milk was as follows: Seven-ounce samples of

* Received for publication April 14, 1915.

1. Jour. Am. Med. Assn., 1912, 58, p. 1848.

2. Ibid., p. 773; p. 1852.

3. Jour. Infect. Dis., 1912, 11, p. 338.

milk were centrifugated at high speed for twenty minutes. The sediment obtained was streaked on the surface of blood agar plates and inoculated into tubes of ascites dextrose broth. Hemolyzing streptococci, similar to those obtained from the throat cultures, were obtained in nine of eleven samples so treated; four of these were virulent for guinea-pigs, producing a fatal peritonitis when injected intraperitoneally.

On the strength of these findings, the health officials required that for a time pasteurized milk only should be sold in Elmhurst. Promptly upon compliance with this order, the epidemic subsided.

Observations on the pathogenicity of Strains 6 and 8, two virulent strains from the milk, will serve to illustrate the results obtained.

Cultures, grown twenty-four hours in ascites dextrose broth, were injected intravenously into healthy, half-grown rabbits. Rabbits 1 and 3 received large inoculations (the growth from 45 c.c. of the broth cultures suspended in 3 c.c. NaCl solution) of Strains 6 and 8, respectively, and Rabbits 2 and 4 received small inoculations (10 c.c.). Both Rabbits 1 and 3 died within one-half hour, apparently from acute intoxication. The bodies were incubated two hours and then cultures were made of the joint fluids of both rabbits. Rabbit 1 gave large numbers of the inoculated organisms in pure culture; Rabbit 3 gave negative results.

Rabbit 2, which received the small inoculation of Strain 6, died in six days. Examination showed polyarthritis, involving each of the legs and three of the lower dorsal vertebrae, also a myocarditis and myositis. The muscles of the chest and shoulders were thickly studded with fine, slender, elongated lesions running parallel with the muscle fibers. These on microscopic examination showed masses of streptococci. The organisms were recovered in pure culture from these lesions and from the blood. A similar result followed upon re-inoculation.

Rabbit 4, inoculated with Strain 8 at the same time and under the same conditions as Rabbit 3, developed symptoms of arthritis. It died on the eighth day following inoculation. The peritoneal surfaces were congested and the peritoneal fluid increased in amount. The gall-bladder was distended and showed numerous whitish patches about 0.5 mm. in diameter. The other organs showed no changes. Cultures from the blood, joints, pericardiac and peritoneal fluids gave negative results. Cultures from the bile gave numerous colonies of a pure culture of the streptococcus inoculated. Sections of the gall-bladder showed numerous small areas entirely denuded of epithelial lining. Cultures of Strain 8, as recovered from the gall-bladder in Rabbit 4, were again inoculated into rabbits. It now caused death in eight days, the rabbit showing marked congestion of the liver and acute peritonitis. The peritoneal fluid contained a pure culture of the organism in large numbers, the blood a few, while the other body fluids were sterile. One rabbit had, in addition to this, a slight pericarditis with increase of pericardiac fluid, and one, a bronchopneumonia of the right lung.

The point of special interest in these experiments is the fact that two strains of streptococci isolated from the milk, indistinguishable from each other as to form and cultural characteristics, were nevertheless quite different, as shown by intravenous injection into animals, one localizing in the muscles and joints, and the other in the gall bladder. It is significant that the strain which produced arthritis was found in large numbers in the joint in one of the rabbits which died one-half hour after injection of the large dose, while the strain which failed to produce arthritis was proved to be absent in the joints in the other animal that died one-half hour after injection.

SUMMARY

An epidemic of streptococcal sore throat, traced to an infected milk supply, subsided when pasteurization of the milk was instituted.

Virulent streptococci isolated from the milk showed selective preference for certain animal structures, such as joints, muscles, gall-bladder, etc., as had the streptococci previously observed by Rosenow⁴ in strains isolated from articular and muscular rheumatism in man, and in certain "laboratory" strains after they had acquired a certain grade of virulence. Furthermore, the organisms from milk resembled the rheumatic strains culturally and morphologically. Involvement of muscles and joints occurred in patients who were infected by the milk. These observations strongly suggest that infected milk, in addition to causing epidemics of sore throat, in which the symptoms are acute and marked, may be the source of streptococci of such virulence as to cause rheumatism and allied conditions in human beings.

4. Jour. Infect. Dis., 1914, 14, p. 61.

PREDOMINANCE AMONG THE MEMBERS OF THE BACILLUS COLI GROUP IN ARTIFICIALLY STORED WATER *

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THE EFFECT OF STORAGE

Up to recent years, the presence of typical *Bacillus coli* in a water supply has been considered as an index to recent pollution. The exponent of this view, Houston, believes that the class of organisms which ferment lactose and produce indol in peptone broth is indicative of recent pollution. It has been shown however by Peckham (1897) that the indol reaction is highly variable. MacConkey (1905) has divided the *Bacillus coli* group into four large sub-groups according to their ability to ferment saccharose and dulcité, a classification which has received almost universal approval. According to MacConkey, and later Jackson (1908), all organisms fermenting lactose may be divided into the four following groups:

Group	Saccharose	Dulcité
<i>Bacillus communior</i>	+	+
<i>Bacillus communis</i>	—	+
<i>Bacillus aerogenes</i>	+	—
<i>Bacillus acidi lactici</i>	—	—

The sign + means gas production.

By far the most complete work on the practical application of this classification to the problem of recency and remoteness of pollution has been carried on by Major W. W. Clemesha.¹ In his work on the highly polluted water supplies of India, he has found that the groups fermenting dulcité, *Bacillus communior* and *Bacillus communis*, are sensitive organisms, found only in numbers where pollution is recent, and that the groups negative to dulcité, *Bacillus aerogenes* and *Bacillus acidi lactici*, are highly resistant forms, which occur in numbers when the pollution has been remote or the water stored for a long time.

In Houston's classification of the *Bacillus coli* group into "Typical and Atypical," the groups fermenting dulcité, *B. communior* and *B.*

* Received for publication April 17, 1915.

1. *Bacteriology of the Surface Waters in the Tropics*, 1912.

communis, which are considered by Clemesha as indicating recent pollution, do not fall into the class of true colon bacilli. This has led to a long discussion. According to Clemesha, "true and atypical coli are purely laboratory classifications; they are not in the slightest degree based on a study of the natural characteristics of fecal organisms, and in estimating the age of a pollution they are of no value." In reviewing Clemesha's work, one must realize that conditions under which he worked were very different from those confronting workers in England and America, and that the hot Indian climate, populated by a people whose diet is very different from ours, offers a different problem so far as the purity of a water supply is concerned.

Stimulated by the discussion between Clemesha and Houston, workers in this laboratory undertook to study the effect of storage on predominance among the various members of the bacillus coli group, according to the MacConkey classification. The routine analyses were carried out by P. Astrofsky and I. Cohen, whom I wish to thank for their co-operation.

In October, 1913, ten one-gram portions of feces from five of the laboratory assistants were emulsified in 1,000 c.c. of tap water in the common one-liter bottles of the reagent type. Each bottle was plugged with cotton to exclude further contamination, while at the same time allowing a free circulation of air. Five of these bottles were placed in a covered box in a dark corner of the laboratory, while the comparative five were placed in a position where light could easily play upon them. The temperature, at all times, was approximately 20-21 C. In February, 1914, another series of five bottles from five other students was similarly stored in the light. At the beginning of each series of experiments, the emulsion of each bottle was well shaken and 1 c.c. and its dilutions were plated on litmus lactose agar and incubated at 37 C. for twenty-four hours, after which the total and bacillus coli counts were made. From the plates representing each bottle, twenty-five to thirty colonies at random were fished onto agar slants, from which inoculations were made into lactose peptone bile as a presumptive test for membership in the bacillus coli group, and into dulcete and saccharose broth to determine their classification according to the MacConkey scheme. This same procedure was followed out each week until the bottles failed to yield any more members of the group. During the course of the experiments, some 4,000 cultures were isolated and studied according to this method. Early in the work it was found that individual labeling was impossible and so elastic bands were used to keep the culture and its corresponding fermentative tubes together, the tubes from each bottle being kept in a separate basket. It may be said that all media were made according to the Standard Methods as proposed by the American Public Health Association. At first, in determining gas production, the regular fermentation tubes were used, but very soon inverted vials were substituted, both for convenience and for ease of cleansing. The lactose peptone bile used as a presumptive test for membership in the bacillus coli group was sterilized in test tubes 5 x 5/8

inches with inverted vials $2 \times \frac{1}{4}$ inches. The dulcitol used for differentiating the cultures according to the MacConkey scheme, as a result of its cost and scarcity, was sterilized in homeopathic vials $2 \times \frac{3}{8}$ inches with the inverted vials $\frac{1}{8} \times 1$ inch. The latter were made in the laboratory by cutting $\frac{1}{8}$ inch glass tubing into lengths of $1\frac{1}{4}$ inches and sealing the end with a blast flame. The saccharose was sterilized in the same manner as the bile. The fermentative tubes were incubated at 37 C. for forty-eight hours, as the maximal gas production always occurred by, or before, that time. Results are expressed as positive or negative gas production, as the author believes that percentages of gas production are of no value, since the reaction, especially of bile, determines the amount of gas produced.

Table 1 gives the initial frequency of various members of the bacillus coli group in the fresh feces, classified according to the MacConkey scheme.

TABLE 1
FREQUENCY OF MEMBERS OF THE BACILLUS COLI GROUP IN FRESH FECES

Number of Sample of Human Feces	Bacillus communior	Bacillus communis	Bacillus aerogenes	Bacillus acidi lactici
1.....	4.3	78.5	0	17.2
2.....	0	100.0	0	0
3.....	0	100.0	0	0
4.....	32.0	20.0	0	48.0
5.....	47.0	35.3	0	17.7
6.....	0	5.0	15.0	80.0
7.....	0	0	6.2	93.8
8.....	0	3.7	3.7	92.6
9.....	0	4.1	0	95.9
10.....	0	0	0	100.0
11.....	7.7	0	7.7	84.6
Summary.....	8.3	31.6	3.0	57.2

Total count of bacteria was 175 organisms.

These results may be compared with the results of other workers.

	Bacillus communior	Bacillus communis	Bacillus aerogenes	Bacillus acidi lactici
MacConkey	23.0	37.0	15.0	25.0
Clemesha	6.8	17.4	22.2	53.2
Winslow and Walker..	28.0	60.0	4.0	8.0
Graham Smith.....	43.0	17.0	11.0	29.0
Browne	8.3	31.6	3.0	57.2

It may be well to state that in this work Samples 1-5 inclusive were examined in October, Sample 6 in December, and Samples 7-11 inclusive in February. The results obtained seem to indicate that there is not only a great individual variation in the flora of the feces, but a seasonal variation as well. Clemesha has pointed out that there are epidemics of certain organisms in the feces. If such is the case, it is highly important to know the conditions governing the presence of the various forms during the various periods.

TABLE 2

THE EFFECT OF STORAGE IN DIFFUSE LIGHT UPON THE PRESENCE OF THE VARIOUS MEMBERS OF THE BACILLUS COLI GROUP (1ST SERIES)

Bacillus Coli Group	Number of Days Stored in the Light									
	0	8	13	20	31	33	47	56	69	73
B. communior.....	16.7	31.9	37.3	26.3	43.5	20.7	38.5	24.9	27.9	23.7
B. communis.....	66.9	41.7	25.0	35.6	31.6	27.6	34.1	39.8	22.6	42.8
B. aerogenes.....	0.0	1.0	21.0	21.6	3.5	14.5	2.3	4.3	8.9	3.1
B. acidi lactici.....	16.4	25.4	16.7	16.5	21.4	37.2	25.1	31.0	40.6	30.4

The percentages represent a summary of the counts made in five samples.

In the study of Table 2, certain results seem to stand out: The percentage of frequency of *Bacillus communior* increases in all five samples during storage, a result out of accord with Clemesha's work, in which he maintains that *Bacillus communior* is a weak organism and never multiplies on storage. *Bacillus communis*, on the other hand, shows a tendency to weaken but is by no means absent at the end of seventy-three days. *Bacillus aerogenes* does not seem to be present in very large numbers in any of the samples. The fourth member of the group, *Bacillus acidi lactici*, shows a tendency to increase during storage. If, however, we examine the total percentage of positive dulcitate fermentation in each bottle at the beginning and at the end of each experiment, we find that the results are nearly alike.

	Percentage of Dulcitate Fermentation—	
	Initial Frequency	Final Frequency
Sample 1.....	84	89
Sample 2.....	100	94
Sample 3.....	100	100
Sample 4.....	52	64
Sample 5.....	82	35

If, as Clemesha states, fermentation of dulcitate represents recent pollution, then these results do not accord with his, for here the percentage of frequency in some cases increases.

In the series of experiments in which the bottles were kept in the dark, an attempt was made to imitate the conditions below the surface of a body of water, and thus see whether light has any effect upon the presence or absence of the various members of the group. As has been stated, these bottles were inoculated with a gram of fresh feces in the same manner as the bottles stored in the light because it had been shown by Clemesha that laboratory cultures behave differently from organisms obtained directly from the intestinal tract.

TABLE 3

THE EFFECT OF STORAGE IN THE DARK UPON THE PRESENCE OF THE VARIOUS MEMBERS OF THE *BACILLUS COLI* GROUP (1ST SERIES)

Bacillus Coli Group	Number of Days Stored in the Dark								
	0	13	20	31	38	47	56	69	73
<i>B. communior</i>	16.7	34.5	56.3	59.9	14.9	26.5	41.5	25.2	38.0
<i>B. communis</i>	66.9	27.5	22.8	26.1	22.3	15.3	42.7	57.7	49.5
<i>B. aerogenes</i>	0.0	14.9	7.7	2.0	36.1	25.1	5.1	3.7	12.5
<i>B. acidi lactici</i>	16.4	23.1	13.2	13.0	26.7	33.1	10.7	27.4	0.0

The percentages represent a summary of the counts made in five samples.

The results in Table 3 seem to substantiate those in Table 2. *Bacillus communior* shows an initial frequency of 16.7 percent, and a final frequency of 38 percent at the end of seventy-three days, an increase of over 100 percent. *Bacillus communis* shows the same gradual decrease as before, and the other two forms reach their highest point midway and decrease toward the seventy-third day. It is interesting that the final positive fermentation of dulcitate is either more than or equal to the initial fermentation of dulcitate.

	Percentage of Dulcitate Fermentation—	
	Initial Frequency	Final Frequency
Sample 6.....	84	100
Sample 7.....	100	100
Sample 8.....	100	100
Sample 9.....	52	75
Sample 10.....	82	63

Tables 2 and 3 indicate that the presence of light has little or no effect upon the presence of the various members of the *bacillus coli* group.

In February, the second series of bottles was inoculated and stored in the room, as the previous experiments had shown that light had little or no effect upon the presence of members of the *bacillus coli* group. This series was conducted along the same lines exactly as the December series and so the two are directly comparable.

TABLE 4

THE EFFECT OF STORAGE IN DIFFUSE LIGHT UPON THE FREQUENCY OF THE VARIOUS MEMBERS OF THE *BACILLUS COLI* GROUP (2ND SERIES)

Bacillus Coli Group	Number of Days Stored in the Light					
	0	7	14	28	35	42
<i>B. communior</i>	1.5	12.3	2.0	23.3	24.8	21.0
<i>B. communis</i>	1.5	5.7	8.8	26.3	15.4	32.2
<i>B. aerogenes</i>	3.5	0.8	4.6	0.0	2.5	2.0
<i>B. acidi lactici</i>	93.5	81.2	84.6	50.4	57.3	44.8

The percentages represent a summary of the counts made in five samples.

Table 4 shows that the results obtained in the second series of bottles substantiate those obtained in the first series: *Bacilli communior* and *communis*, which, according to Clemesha, are supposed to be the least resistant forms, increase in frequency many fold. *Bacillus aerogenes* plays the same inert part. *Bacillus acidi lactici*, according to Clemesha the more resistant form, decreases in frequency.

THE EFFECT OF ENRICHMENT AND STORAGE

During the second series of experiments (in February), a comparative test was made to see whether enrichment in lactose peptone bile had any effect upon predominance among the members of the *Bacillus coli* group. The procedure was the same as that described above except that, at the time when the direct litmus lactose agar plates were made from the fecal emulsions, lactose peptone bile tubes were inoculated with 1 c.c. of the emulsion and incubated for twenty-four hours at 37.5 C. Litmus lactose agar plates were made from the bile tubes and cultures isolated as in the direct plating. The procedure may be illustrated as follows:

	Not Enriched	Enriched
Feb. 17	Various dilutions from each bottle plated on litmus lactose agar plates, and incubated at 37.5 C. twenty-four hours.	One c.c. from each bottle enriched in lactose peptone bile and incubated at 37.5 C. twenty-four hours.
Feb. 18	One hundred and twenty-five colonies fished from five bottles onto agar slants, and incubated at 37.5 C. twenty-four hours.	The enriched cultures plated on litmus lactose agar plates and incubated at 37.5 C. twenty-four hours.
Feb. 19		One hundred and twenty-five colonies fished from five bottles onto agar slants and incubated at 37.5 C. twenty-four hours.
Feb. 21	Inoculation from each streak made into dulcitate and saccharose broth, and lactose peptone bile; incubated at 37.5 C. seventy-two hours.	Each streak inoculated into dulcitate and saccharose broth and lactose peptone bile and incubated at 37.5 C. seventy-two hours.
Feb. 24	Recorded gas.	

Other conditions not mentioned here are exactly as stated in the first part of the paper.

TABLE 5

THE EFFECT OF ENRICHMENT IN LACTOSE PEPTONE BILE UPON PREDOMINANCE AMONG THE MEMBERS OF THE *BACILLUS COLI* GROUP ISOLATED FROM STORED WATERS

Bacillus Coli Group	Number of Days Stored											
	0		7		13		27		34		41	
	a	b	a	b	a	b	a	b	a	b	a	b
B. communior.....	1.5	1.6	12.3	8.9	2	4.6	23.3	24.1	24.8	17.3	21	15.9
B. communis.....	1.5	5	5.7	15.4	8.8	0.7	26.3	17.7	15.4	7.8	32.2	27.2
B. acidi lactici.....	93.5	90.6	81.2	72.3	84.6	91.6	50.4	57.2	57.3	72.4	44.8	56.0
B. aerogenes.....	3.5	2.5	0.8	3.2	4.6	3.1	0	0.8	2.5	2.3	2.0	0.7

The percentages represent a summary of the counts made in five samples.

a = not enriched in lactose peptone bile.

b = enriched in lactose peptone bile.

The results of these experiments, as shown in Table 5, corroborate those brought out earlier in the paper.

CONCLUSIONS

A comparison of the feces taken during different periods indicates a differentiation in the flora of the bacillus coli group, as evidenced by the fermentation of dulcitol and saccharose.

Bacillus communior, a dulcitol-fermenting organism, which has been regarded as an index to recent contamination, not only holds its own, but increases in prevalence during storage.

Bacillus communis, the other dulcitol-fermenting member of the bacillus coli group, also regarded as an index to recent contamination, altho it rarely ever shows an increase in prevalence during storage, nevertheless decreases very gradually and slowly.

The frequency of fermentation of dulcitol by organisms isolated from fresh feces, and that by organisms from fecal emulsions stored seventy-three days, approximate each other very closely.

The presence of light or darkness does not seem to affect the ratio of the four groups.

Enrichment in lactose peptone bile does not seem to favor the growth of one member of the bacillus coli group over the others; for, in comparative tests, those strains which were enriched in lactose peptone bile appeared in the same percentage of frequency as those strains which were not enriched.

A METHOD OF TRANSMITTING KNOWN NUMBERS OF TRYPANOSOMES WITH A NOTE ON THE NUMERIC RELATION OF TRYPANOSOMES TO INFECTION *

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This method for transmitting trypanosomes was evolved during chemotherapeutic researches in which several species of these parasites were used as the test organism, by reason of the necessity for having a fairly accurate, rapid, and simple means of infecting rats with known numbers of trypanosomes. As will be pointed out later in this paper, the time of true incubation of trypanosomes following infection by the intraperitoneal route, as well as to some extent the duration of the animal's life, are factors influenced by the numbers of trypanosomes injected; or, even more important, as will be discussed in a later paper, is the fact that the parasitropic effects of a drug or substance under study and the resistance of the animal to the drug's organotropic effects are apparently modified, to some extent at least, by whether the animal was infected with small or large numbers of trypanosomes. While it is necessary to use a parasite of sufficient aggressiveness and vitality to yield definite results and indicate any therapeutic action of the agent under study, yet it is possible to mask the finer grades of therapeutic effects by an overwhelming infection, or, on the other hand, lose or vitiate an experiment by infecting with too few parasites.

With these practical considerations in mind, I have worked out a method for transmitting approximately known numbers of trypanosomes, which has proved comparatively simple, fairly accurate, and very useful. By this method, it is possible to infect a large series of rats with any desired number of trypanosomes, in a sterile manner, and without sacrificing the seed animal. It is highly desirable, especially in chemotherapeutic researches, to infect the animals with a uniform number of parasites, not only the animals of a single series, but likewise those of different series, and this method has been found to fulfill this important requirement satisfactorily.

* Received for publication April 26, 1915.

Briefly, the method consists in securing a definite volume of blood in a blood corpuscle-counting pipet and mixing it with a definite amount of a diluting fluid that hemolyses the erythrocytes, and fixes and stains the trypanosomes. The trypanosomes are then counted by means of a Thoma-Zeiss counting chamber and the number in a cubic centimeter of undiluted blood calculated. With this data at hand, 0.1 c.c. of blood is drawn aseptically from the heart of the seed animal in 0.9 c.c. of a 10 percent solution of sodium citrate in normal salt solution. With this blood, dilutions are made in warm, sterile, normal salt solution until a cubic centimeter or a fraction thereof contains the required dilution and number of trypanosomes determined upon for the purpose of infecting a series of rats, or for any other purpose.

TECHNIC

Seed Animal.—For the purpose of making an accurate count, the seed animal should be one that has not been infected over three or four days. Especially is this true of rats infected with *Trypanosoma brucei* and *Trypanosoma equiperdum*, as after this time the parasites in the peripheral blood may be very numerous and, what is more important, may be gathered in conglomerated masses which cannot be broken up in the mixing pipet and which render any attempts at counting absolutely worthless. With rats infected with *Trypanosoma lewisi*, older infections than those mentioned may be found satisfactory, as the number of parasites is not usually so great as found with the more pathogenic species, and conglomeration is not so likely to occur.

A preliminary examination of the blood is first made in the usual manner by snipping off the tip of the tail and quickly transferring a drop of the blood on a clean cover glass to a microscopic slide. The weight of the glass is allowed to spread the blood. This preparation is then examined by means of a No. 1/6" objective and No. 4 eyepiece (Leitz), and the number of trypanosomes recorded according to the following scheme:

- ++++ = very large numbers; cannot be counted.
- +++ = large numbers that may be roughly counted (not accurately).
- ++ = about 10-20 in a field.
- +
- +
- +
- Few = 1-2 in a field, or in every other field.

This preliminary examination is necessary because it indicates the blood pipet to use, as the red corpuscle pipet for a 1:200 dilution with ++++ and +++ rats, and the white corpuscle pipet for a 1:20 dilution with ++ and + rats. This method of recording the count of trypanosomes has been found useful as a routine procedure in the daily examination of large numbers of rats in our chemotherapeutic researches and with due care it yields a satisfactory record.

The Diluting Fluid.—After some experimentation a fluid has been adopted which serves the three-fold purpose of (1) fixing the trypanosomes and rendering them motionless, (2) quickly staining the trypanosomes so that they are readily seen and counted, and (3) removing the erythrocytes by hemolysis so

that the parasites are readily seen in a clear field. It is easily prepared and comparatively stable, remaining clear, without sediment, for many weeks and months at ordinary room temperature.

This fluid is prepared as follows:

Formalin (40 percent)	2 c.c.
Glacial acetic acid	2 c.c.
Distilled water	96 c.c.

Mix and add 2 c.c. carbolfuchsin (Ziehl-Neelson); mix again and filter through paper. This quantity of fuchsin in the presence of formalin gives the fluid a clear, deep-purplish red color. Probably less fuchsin will prove satisfactory to others; more of the dye deepens the color of the fluid unnecessarily and may somewhat obscure the lines of the counting chamber. The formalin quickly destroys the motility of trypanosomes and keeps the fluid free of bacteria and fungi. The container should be well stoppered to prevent evaporation, and it may be advisable to refilter the fluid at occasional intervals.

The Counting of the Trypanosomes.—After the seed animal has been selected, a count is made of the trypanosomes. The tail is gently whipped back and forth for a minute or two to produce congestion; then, upon very gentle pressure, a large drop of blood appears where the tip has been snipped off. With the red corpuscle pipet, blood is carefully drawn to the mark 0.5 and the diluting fluid to the mark 101, the whole procedure being conducted with the care and exactness required for making an erythrocyte count. If the white corpuscle pipet is used, a large drop of blood is required and hence, before attempting to fill the pipet, a free flow of blood must be assured. With this pipet, blood is drawn to the mark 0.5 and the diluting fluid to the mark 11, as in the usual technic for counting leukocytes. As a general rule, the red blood corpuscle pipet is indicated and is more satisfactory than the white blood corpuscle pipet.

The filled pipet is now thoroughly shaken for at least one or two minutes; several drops are expelled, and then sufficient fluid is placed in the chamber and the cover glass accurately adjusted so that there is no fluid in the moat and Newton's color rings are seen. I never drop the cover glass on the chamber, but prefer to slide it quickly into place, firmly pressed to the slide. The slide is allowed to rest on a flat surface for at least ten minutes to permit the trypanosomes to sink down on the ruled off spaces.

By means of the No. 1/6" objective and No. 4 or No. 2 eyepiece (Leitz) and a mechanical stage, all the trypanosomes in the ruled off space (400 small squares) of the Thoma ruling are counted. The trypanosomes are motionless, deeply stained a purplish red, and easily seen and counted. At times they are curled or placed edgewise, but with a clean diluting fluid, to avoid artefacts, and a little experience these forms are readily recognized. The red blood corpuscles have entirely disappeared, or appear as faint shadows. The leukocytes also are deeply stained, and a total leukocyte count may be made at the same time. A differential leukocytic count is also possible, at least as far as differentiating between the polynuclear and mononuclear leukocytes (Fig. 1).

In counting, it is well to start in the upper left hand corner and move across to the right, returning to the left, and so on, until the 400 small squares have been counted. If many trypanosomes are present, it is well to note on paper the number found in each of the four large squares across the ruled off space including the double lines; if few are present, the whole ruled off space may be counted in one figure. Trypanosomes placed half or more within the lines are included; otherwise, they are not included in the count. As a general rule,

it is well to go over the slide twice; the totals should not differ by more than one or two trypanosomes and the counts of different persons should give the same tally and usually do after brief experience. These counts are more accurate than those ordinarily made of human erythrocytes and in every way as accurate as a leukocytic count made with equal care.

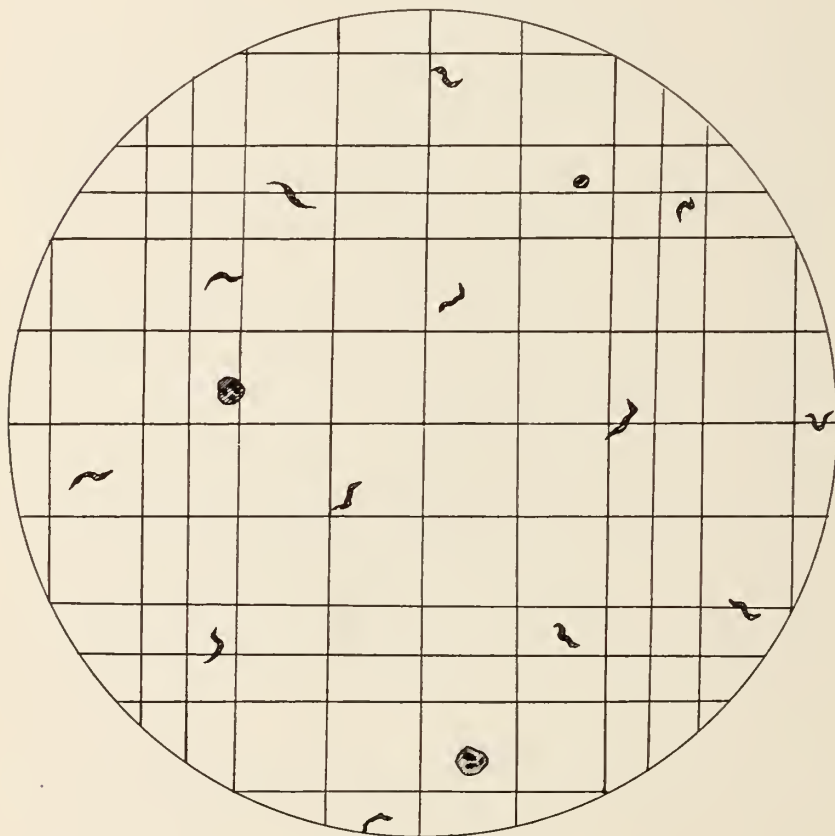


Fig. 1.—A section of the four hundred small squares of the Thoma ruling, showing trypanosomes in blood, diluted 1:200 (182,000,000 per cubic centimeter of undiluted blood), from a + + + + rat infected with *Trypanosoma lewisi*. X 288 (Leitz No. 6, eyepiece No. 2)

The Calculation of the Number of Trypanosomes in a Cubic Centimeter Undiluted Blood.—As each square contains $1/4000$ cubic millimeter when the cover glass is properly adjusted, it is necessary to count the trypanosomes in 4,000 small squares to obtain the number contained in 1 cubic millimeter of the diluted blood. By counting the trypanosomes in 400 small squares and multiplying by 10, the approximate number in 4,000 small squares, or 1 cubic millimeter, is obtained. By multiplying this figure by the dilution as 200, if the red blood corpuscle pipet was used, or by 20 in case the white blood corpuscle pipet

was used, the number in 1 cubic millimeter of undiluted blood is obtained. Multiplying this figure by 1,000 gives the approximate number of trypanosomes in a cubic centimeter of undiluted blood. An example briefly illustrates the method:

42	trypanosomes in ruled off area (400 small squares)
$\times 10$	
<hr/>	
420	trypanosomes in 4,000 small squares, or in one cubic millimeter of
$\times 200$	blood diluted 1:200
<hr/>	
84,000	trypanosomes in one cubic millimeter of undiluted blood
$\times 1,000$	
<hr/>	
84,000,000	trypanosomes in one cubic centimeter of undiluted blood

Briefly, by rule of thumb, count the trypanosomes in 400 small squares, multiply by 200, and add four ciphers. The result is the approximate number of trypanosomes in a cubic centimeter of undiluted blood.

If the white corpuscle pipet was used, the calculations are the same, except that the dilution is 1:20, as illustrated in another example:

93	trypanosomes in 400 small squares
$\times 10$	
<hr/>	
930	trypanosomes in 4,000 small squares, or one cubic millimeter of
$\times 20$	blood diluted 1:20
<hr/>	
18,600	trypanosomes in one cubic millimeter of undiluted blood
$\times 1,000$	
<hr/>	
18,600,000	trypanosomes in one cubic centimeter of undiluted blood

Or, by rule of thumb, count the trypanosomes in 400 small squares, multiply by 20, and add four ciphers.

A larger number of squares may be counted after a well known method for counting leukocytes; namely, by adjusting the microscope in such manner that the diameter of the field is just exactly eight small squares when the entire field contains fifty squares ($\pi r^2 = 3.1416 \times 16 = 50.26$); by counting the trypanosomes in twenty such fields, 1,000 small squares are covered, and multiplying this number by 80 gives the number of trypanosomes in 80,000 small squares, or one cubic millimeter of undiluted blood, and this figure multiplied by 1,000 gives the number of parasites in a cubic centimeter of undiluted blood. In the first method, 400 small squares are counted and in this method, 1,000 squares; comparative counts have been found to tally closely.

I have also tried the method of counting trypanosomes which consists in preparing a smear of the undiluted blood directly from the tail on a microscopic slide or on cover glasses, fixing with methyl alcohol, and staining with a suitable dye, as 1:10 carbolfuchsin, and then counting the trypanosomes and red blood corpuscles after the same manner in which Wright counts a bacterial vaccine. This method is relatively crude, yielding too variable results in different smears from the same rat prepared at the same time. The trypanosomes are frequently distributed in an irregular manner, and the number of eryth-

rocytes in a cubic millimeter of normal rat blood is too valuable to adopt that figure as reliable which forms the basis of this method.

A question of considerable importance in this connection is whether counts of trypanosomes made with blood from the tail may be accepted as reliable and similar to counts made with blood from the heart, especially as blood for the purpose of transmitting the parasites is obtained from the heart of the seed animal.

I have made a series of counts of blood obtained from the tail, and from the right and left ventricles of the heart. The results of five of these comparative counts are shown in Table 1.

TABLE 1
COMPARATIVE COUNTS OF TRYPANOSOMES IN BLOOD FROM THE TAIL AND FROM THE HEART
OF RATS

Rat	Infection	Direct Examination	Count of Blood from:		
			Tail	Right Ventricle	Left Ventricle
1	T. lewisi.....	++++	42,000,000	44,000,000	42,000,000
2	T. lewisi.....	++++	44,000,000	44,000,000	30,000,000
3	T. lewisi.....	++++	48,000,000	50,000,000	46,000,000
4	T. equiperdum.....	++++	1,400,000,000	1,360,000,000	1,200,000,000
5	T. lewisi.....	++++	40,000,000	41,000,000	40,000,000

As will be noted, the counts were found to run fairly parallel, especially in view of the large number of trypanosomes present and the chances of manipulative error. Frequently counts of blood obtained from the left ventricle showed slightly fewer trypanosomes than those of blood from the right ventricle and tail, but on the whole they were sufficiently close for the purpose of counting trypanosomes in the blood from the tail and using this figure as representing the number present in blood from the heart in the transmission of approximately known numbers of trypanosomes. It must be emphasized, however, that blood from the tail must flow easily and freely before counts are reliable; those from blood obtained after squeezing and massage of the tail are inaccurate and unreliable.

The Securing of Infected Blood for Transmissions.—Blood is obtained from the heart of the seed animal after the method I have described in a previous paper.¹ In this way, 0.1 c.c. of blood may be obtained in an accurate and sterile manner, the success of this method of transmitting known numbers of trypanosomes depending of course upon the operator's success in obtaining the blood accurately and quickly from the heart. There must be no more and no less blood, and the dilutions must be accurate.

This amount of blood is easily obtained from the living rat. If it cannot be drawn accurately, the animal should be killed, the thorax opened, and the blood drawn directly from the heart under the eye of the operator. With a little practice, however, one seldom fails to draw the blood from the living rat, and usually the operation does not appear to injure the animal.

It is necessary to use an accurate and well adjusted 1 c.c. syringe divided into tenth cubic centimeters. I prefer the Record syringe fitted with a No. 22 needle. A proper needle is very important; it must be sharp and sufficiently large to permit the easy flow of blood and yet not so large as unnecessarily to injure the heart.

(1) Warm a test tube containing 10 percent sodium citrate in normal salt solution in a Bunsen burner and draw up enough of this into the syringe to make, when air has been expelled, 0.4 c.c., including the needle. (The sodium citrate prevents coagulation of the blood under isotonic conditions.)

(2) The rat being held by an assistant, or fastened to an operating board, paint the left thorax with tincture of iodine and quickly insert the needle at about the point of maximal pulsation. By gentle suction on the piston, blood is made to flow into the syringe. If blood does not appear, re-insert the needle in an upward and inward direction without withdrawing it entirely. Draw blood accurately to the 0.5 c.c. mark (\approx 0.1 c.c. blood). Withdraw the needle and draw more of the warm citrate solution to the 1 c.c. mark. This gives a dilution of blood in the syringe equal to 1 in 10. Shake gently in order to mix the blood.

(3) Expel the contents of the syringe into a test tube containing 9 c.c. of warm salt solution and wash out the syringe three or four times with this solution, or the contents of the syringe may be expelled into a tube containing 6 c.c. of warm normal salt solution and the syringe washed out with 3 c.c. fresh warm salt solution into this tube in order better to remove all trypanosomes from the syringe and needle. This gives a suspension of 1:100, and from this tube further suspensions of the trypanosomes may be made in warm, sterile salt solution as required.

The Dilution of Infected Blood for Transmissions.—A 1:100 suspension of infected blood having been secured, further suspensions may be made by transferring 1 c.c. to a series of test tubes containing 9 c.c. of warm, sterile salt solution. The 1:100 suspension and each succeeding one should be thoroughly mixed by furnishing each test tube with a sterile rubber stopper and then gently agitating the contents with turning up and down until a thorough admixture results. With the final suspensions, a series of rats may be infected by injection of each intraperitoneally with a cubic centimeter or fraction thereof, according to the count. An example will explain this step:

If a count of 84,000,000 trypanosomes (*equiperdum*) in a cubic centimeter of undiluted blood has been found, 0.1 c.c. blood + 0.9 c.c. citrate solution (No. 1) + 9 c.c. normal salt solution equals a 1:100 suspension (No. 2), each cubic centimeter of which contains 840,000 trypanosomes, or 0.1 c.c. contains 84,000 trypanosomes.

One c.c. of this suspension (No. 2) in 9 c.c. of normal salt solution gives a suspension (No. 3) of which 1 c.c. contains 84,000 trypanosomes. Rats may then be injected intraperitoneally with 1 c.c. of this suspension (84,000)—a procedure which is probably better than the injection of 0.1 c.c. of Suspension 2. Further dilutions may be made and fewer trypanosomes injected by using fractions of a cubic centimeter, as 0.5 c.c., in this instance containing 42,000 trypanosomes, or 0.2 c.c., containing 16,800, etc.

In conducting chemotherapeutic researches with *T. equiperdum*, best results have been obtained when the rats were infected with about 100,000 trypanosomes an animal. With *T. brucei* fewer may be used and with *T. lewisi* a larger number should be injected.

The Method of Infecting Rats.—Rats may now be infected by the intraperitoneal injection of the proper dose of trypanosome suspension. For this purpose, I prefer a 1 c.c. Record syringe fitted with a No. 22 needle. The suspension of trypanosomes should be briefly and gently mixed each time before the syringe is loaded in order to avoid settling. The syringe should be sterile and the technic fairly aseptic, altho I have never seen infection of the peri-

toneum follow when the rat was properly held, a touch of alcohol applied to the abdominal skin, and the needle properly entered. I have not found it necessary to clip the hair or sterilize the skin by washing or even by an application of tincture of iodine, and with due care the intestines are not punctured.

A NOTE ON THE NUMERIC RELATIONS OF TRYPANOSOMES
TO INFECTION

Mainly for the purpose of testing the reliability of this method for transmitting trypanosomes, a number of experiments were conducted with *T. lewisi*, *T. equiperdum*, and *T. brucei*, consisting in the injection of a series of rats with increasing numbers for the purpose of determining three factors: (1) With how few trypanosomes of these three species rats may be infected; (2) the relation of the numbers of trypanosomes injected to the time of true incubation; (3) the relation of the number of trypanosomes injected to the age and duration of life of the infected animal.

Materials.—The three species of trypanosomes were obtained from the Rockefeller Institute through the kindness of Dr. Wade H. Brown. *Trypanosoma lewisi* at times developed a virulence for our albino rats, especially after a number of short interval transfers, an experience with this strain which is similar to that described by Jürgens² and Brown.³

Trypanosoma equiperdum, when injected in very large numbers, usually killed its host about the sixth to the tenth day and *Trypanosoma brucei* about the third to fifth day. These intervals were on several occasions considerably shortened by a series of short interval transfers.

The Method of Study.—The trypanosomes were counted after the method described and increasing numbers injected intraperitoneally into several series of rats, which were numbered and weighed. Daily examinations were made by the usual method of snipping the tip of the tail and directly examining a drop of blood on a cover glass and slide by means of a No. 6 objective and No. 4 eyepiece (Leitz). The number of trypanosomes were recorded after the method of + + + +, + + +, + +, +, and "few," already described. If no trypanosomes were found in at least ten consecutive fields, the result was recorded as negative. In all instances, large, freely flowing drops of blood were examined; blood obtained by undue squeezing and milking of the tail, as stated before, is unreliable.

The results of some of the experiments are shown in the following groups of tables with a summary accompanying each group.

TRYPANOSOMA LEWISI (TABLES 2, 3, 4, AND 5)

(1) The smallest number of *T. lewisi* found capable of infecting a rat was 138 (Table 5); the animal was full grown and weighed 138 grams. The same number injected into a rat of lighter weight failed to infect. The next lowest number was 190, in a rat weighing 46 gm. (Table 2).

2. Arch. f. Hyg., 1902, 42, p. 265.

3. Jour. Exper. Med., 1914, 19, 406; *ibid.*, 1915, 21, p. 345.

TABLE 2
TRYPANOSOMA LEWISI *

No. of Rat	Weight, in Grams	Number of Trypanosomes Injected Intra-peritoneally	Results of Examination of Blood from Tail										
			24 hr.	36 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.
1	52	95	—	—	—	—	—	—	—	—	—	—	—
2	46	190	—	—	—	—	—	—	—	—	—	—	—
3	50	420	—	—	—	—	—	—	—	—	—	—	—
4	53	1,680	—	—	—	—	—	—	—	—	—	—	—
5	109	4,200	—	—	—	—	—	—	—	—	—	—	—
6	136	8,400	—	—	—	—	—	—	—	—	—	—	—
7	115	42,000	—	—	—	—	—	—	—	—	—	—	—
8	125	84,000	—	—	—	—	—	—	—	—	—	—	—

* Seed rat showed + + + + : 42 trypanosomes in 400 small squares (dilution 1:200) = 84 000,000 trypanosomes in 1 cubic millimeter undiluted blood, and 84,000,000 in 1 cubic centimeter.

TABLE 3
TRYPANOSOMA LEWISI *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail															
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.	11 da.	12 da.	13 da.	14 da.	15 da.	16 da.
1	67	186	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	73	1,116	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	62	7,440	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
4	60	14,880	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+
5	62	18,600	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+
6	54	93,000	—	—	—	—	+	+	+	+	+	+	+	+	+	+	+	+
7	73	186,000	—	—	—	—	—	—	—	—	—	—	—	+	+	+	+	+

* Seed rat showed + + : 93 trypanosomes in 400 small squares (dilution 1:20) = 18,600 trypanosomes in 1 cubic millimeter undiluted blood, and 18,600,000 in 1 cubic centimeter.

TABLE 4
TRYPANOSOMA LEWISI *

No. of Rat	Weight In Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail											
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.	11 da.	12 da.
1	56	300	—	—	—	—	—	Few	+	++	++	++	++	++
2	70	1,500	—	—	—	—	—	Few	++	++	++	++	++	++
3	82	3,000	—	—	—	—	—	Few	++	++	++	++	++	++
4	91	15,000	—	—	—	—	—	—	+	++	++	0	++	++
5	102	30,000	—	—	—	—	—	—	—	++	++	—	++	++
6	83	60,000	—	—	—	—	—	—	—	—	—	0	0	0
7	96	150,000	—	Died	0	0	0	0	0	Died	0	0	0	0
8	72	3,000,000	—	—	—	—	—	+	++	++	++	++	++	++

* Seed rat showed + + +: 15 trypanosomes in 400 small squares (dilution 1:200)=30,000 trypanosomes in 1 cubic millimeter undiluted blood, and 30,000,000 in 1 cubic centimeter.

TABLE 5
TRYPANOSOMA LEWISI *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail														
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.	11 da.	12 da.	13 da.	14 da.	15 da.
1	97	138	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	138	138	—	—	—	—	—	—	Few	+	+	+	+	+	+	+	+
3	128	690	—	—	—	Few	+	+	+	+	+	+	+	+	+	+	+
4	83	1,380	—	—	—	Few	+	+	+	+	+	+	+	+	+	+	+
5	120	13,800	—	—	—	Few	+	+	+	+	+	+	+	+	+	+	+

* Seed rat showed + + +: 69 trypanosomes in 400 small squares (dilution 1:20)=13,800 trypanosomes in 1 cubic millimeter undiluted blood, and 13,800,000 in 1 cubic centimeter.

Trypanosoma lewisi under ordinary conditions is to be regarded as feebly aggressive and pathogenic for the majority of albino rats, so that, as a rule, more of these trypanosomes are required to induce uniform infection in fresh animals than are required of *T. equiperdum* and *T. brucei*. Sometimes relatively large numbers failed to infect normal animals, an example being found in Table 4, Rat 6, weighing 83 gm., which the injection of 60,000 trypanosomes did not infect to the extent that the trypanosomes could be found in the peripheral blood up to the ninth day, when it died. In our experience, at least 100,000 trypanosomes should be injected intraperitoneally in order to secure uniform infections.

(2) The time of true incubation of *Trypanosoma lewisi* did not bear any constant or striking relation to the number of trypanosomes injected. In general, the period of incubation was found to be about five days. As will be noted in Table 2, the injection of 1,680 trypanosomes into the peritoneal cavity of a rat weighing 53 gm. was followed by a four day period of incubation, the same interval following the injection of 84,000 trypanosomes into a rat of 125 gm. weight; it must be noted that the second rat was more than twice as heavy as the first. But a similar result is noted in Table 3; in this example an injection of 7,440 trypanosomes into a rat weighing 62 gm. was followed by the same five day incubation period as followed the injection of 93,000 trypanosomes into a rat weighing 54 gm.; in this same series, the injection of 186,000 trypanosomes into a rat of 73 gm. weight (Rat 7) was followed by a twelve day incubation period. Similar examples are found in Tables 4 and 5.

It is to be remembered however that the largest of these doses did not contain nearly as many trypanosomes as are injected by the ordinary technic, especially when undiluted, defibrinated blood is used. With such technic, the time of true incubation is about three or four days and, indeed, adult forms of the trypanosomes may be found in the blood within twenty-four hours, an example of the so-called "false" incubation.

(3) With regard to the relation of the size of the infecting dose to the weight of the animal and to the period of incubation, this series was too short to warrant a conclusion, but, in general, it may be stated that the younger the rat the more subject it is to infection.

Individual resistance of the rat influenced *Trypanosoma lewisi* to a far greater extent than was evident with *T. equiperdum* and especially *T. brucei*.

TABLE 6
TRYPANOSOMA EQUIPERDUM *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail											
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.	11 da.	12 da.
1	76	6	—	—	—	—	—	—	—	—	—	—	—	—
2	56	36	—	—	—	—	—	—	—	—	—	—	—	—
3	71	72	—	—	—	—	—	—	—	—	—	—	—	—
4	59	288	—	—	—	—	—	—	—	—	—	—	—	—
5	73	243	—	—	—	—	—	—	—	—	—	—	—	—
6	108	486	—	—	—	—	—	—	—	—	—	—	—	—
7	109	2,430	—	—	—	—	—	—	—	—	—	—	—	—
8	98	4,860	—	—	—	—	—	—	—	—	—	—	—	—
9	115	24,300	—	—	—	—	—	—	—	—	—	—	—	—
10	112	48,600	—	—	—	—	—	—	—	—	—	—	—	—
11	117	243,000	—	—	—	—	—	—	—	—	—	—	—	—
12	134	486,000	—	—	—	—	—	—	—	—	—	—	—	—

* Szed rat showed + + + + + : 243 trypanosomes in 400 small squares (dilution 1:200) = 486,000 trypanosomes in 1 cubic millimeter undiluted blood, and 486,000,000 in 1 cubic centimeter.

TABLE 7
TRYPANOSOMA EQUIPERDUM *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail									
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	
1	74	2,400	—	—	—	Few	++	++	++	++	Died	++
2	75	4,800	—	—	—	Few	+++	+++	+++	+++	0	+++
3	70	14,400	—	—	—	+	+	+	+	+	Died	+
4	65	24,000	—	—	—	+	+	+	+	+	0	+
5	72	38,400	—	—	—	+++	+++	+++	+++	+++	0	+++
6	66	48,000	—	—	—	+++	+++	+++	+++	+++	0	+++
7	66	144,000	—	—	—	+++	+++	+++	+++	+++	0	+++
8	70	240,000	—	—	—	+++	+++	+++	+++	+++	0	+++
9	95	384,000	—	—	—	+++	+++	+++	+++	+++	0	+++
10	60	480,000	—	—	—	Died	0	0	0	0	0	0

* Szed rat showed + + + : 23 trypanosomes in 400 small squares (dilution 1:200) = 4,800 trypanosomes in 1 cubic millimeter undiluted blood, and 4,800,000 in 1 cubic centimeter.

The death rate among rats infected with this strain of *Trypanosoma lewisi* was variable, without particular relation to the weight of the animal or the number of trypanosomes injected. Most of these experiments were conducted at a time when the strain had an exalted virulence, so that many of the animals succumbed within twenty days after infection.

TRYPANOSOMA EQUIPERDUM (TABLES 6 AND 7)

(1) The smallest number of *T. equiperdum* found to infect a rat was 243 in an animal weighing 73 gm. (Rat 5, Table 6), trypanosomes being found in the blood on the seventh day. Probably a more extensive series of experiments would discover rats that could be infected with a smaller number, as was found with *Trypanosoma lewisi*. In our chemotherapeutic work, we regularly inject our rats with from 80,000 to 100,000 of these trypanosomes in order to insure uniform infections.

(2) Some relation was found between the number of trypanosomes injected and the time of true incubation. As noted in Table 7, the injection of 144,000 and more trypanosomes was followed by a forty-eight-hour period of incubation; the injection of 15,000 to 48,000 was followed by a seventy-two-hour incubation, and the injection of smaller numbers, as 2,400-4,800 and 500-4,860, was followed by periods of four and five days, respectively, before trypanosomes could be found in the peripheral blood.

(3) There was found no constant relation between the number of trypanosomes injected and the duration of life under the conditions of these experiments. While the period of incubation was lengthened by injecting a small number of trypanosomes, the duration of life was in general the same, after trypanosomes were found in the peripheral blood, as when larger numbers were injected. Occasionally rats died on the third day after trypanosomes were found in the blood, but usually the time of death varied from the fifth to the eighth day.

TRYPANOSOMA BRUCEI (TABLES 8, 9, AND 10)

(1) In our experiments, the smallest number of *T. brucei* found capable of infecting a rat was 37 in an animal weighing 95 gm., the parasites appearing in the blood after an eight day period of incubation (Rat 1, Table 8). The injection of as few as 150 of these trypanosomes infected rats weighing 84 and 76 gm. respectively, in two different experiments (Rat 1, Table 9 and Rat 5, Table 10).

TABLE 8

TRYPANOSOMA BRUCEI *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail											
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.	11 da.	12 da.
1	95	37	—	—	—	—	—	—	—	—	+	+++	+++	Died
2	106	74	—	—	—	—	—	—	—	—	—	—	—	0
3	107	370	—	—	—	—	—	—	—	—	—	—	—	0
4	117	740	—	—	Few	+++	+++	+++	0	0	0	0	0	0
5	113	1,480	—	—	+++	+++	+++	Died	0	0	0	0	0	0
6	78	4,440	—	+++	+++	+++	+++	Died	0	0	0	0	0	0
7	69	7,400	—	+++	+++	+++	+++	+++	0	0	0	0	0	0
8	45	14,800	—	—	Few	+	+	+++	0	0	0	0	0	0

* Seed rat showed + + + : 37 trypanosomes in 400 small squares (dilution 1:200) = 74,000 trypanosomes in 1 cubic millimeter undiluted blood, and 74,000,000 in 1 cubic centimeter.

Trypanosoma brucei was found the most aggressive and virulent of the three species of trypanosomes studied, the injection of small numbers being followed most regularly by uniform infections.

(2) A relation was found between the number of trypanosomes injected and the period of incubation.

In general, this relation was found most constant with these trypanosomes, somewhat so with *Trypanosoma equiperdum*, and least with *Trypanosoma lewisi*, having in this manner some bearing on the aggressiveness of these various parasites for the rat.

TABLE 9
TRYPANOSOMA BRUCEI *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail						
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.
1	84	150	—	—	—	—	++	++++	Died
2	93	180	—	—	—	+++	++++	++++	Died
3	88	600	—	—	Few	++	++++	++++	Died
4	72	1,500	—	—	Few	+++	++++	++++	Died
5	66	3,000	—	—	+	++++	++++	++++	Died
6	98	9,000	—	Few	+	++++	Died	0	0
7	70	15,000	—	+	+	++++	++++	++++	Died
8	76	30,000	—	—	++	++++	++++	++++	Died

* Seed rat showed +++: 15 trypanosomes in 400 small squares (dilution 1:200) = 30,000 trypanosomes in 1 cubic millimeter undiluted blood, and 30,000,000 in 1 cubic centimeter.

TABLE 10
TRYPANOSOMA BRUCEI *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail						
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.
1	70	2	—	—	—	—	—	—	—
2	78	8	—	—	—	—	—	—	—
3	79	40	—	—	—	—	—	—	—
4	98	80	—	—	—	—	—	—	—
5	76	149	—	—	—	Few	+	++++	Died
6	92	298	—	—	—	+	++++	++++	Died
7	80	1,490	—	—	Few	++	++++	Died	0
8	85	2,980	—	—	+	++++	++++	++++	Died
9	91	14,900	—	—	+	++++	Died	0	0
10	77	29,800	—	Few	+++	++++	++++	++++	Died
11	110	149,000	—	+	++++	Died	0	0	0
12	82	298,000	—	+	++	++++	++++	++++	Died

* Seed rat showed +++: 149 trypanosomes in 400 small squares (dilution 1:20) = 29,800 trypanosomes in 1 cubic millimeter undiluted blood, and 29,800,000 in 1 cubic centimeter.

After injecting very large numbers of *T. brucei*, as contained, for example, in 0.2-0.3 c.c. of undiluted, defibrinated blood, I have on occasion found trypanosomes in the peripheral blood in twenty-four hours.

The intraperitoneal injection of about 5,000 (Rat 6, Table 8) to about 300,000 (Rat 12, Table 10) trypanosomes was followed by a period of incubation of about forty-eight hours; possibly prolonged examinations at more frequent intervals would have shown an earlier appearance of the parasites in the peripheral blood with the larger doses. The injection of about 600 (Rat 3, Table 9) to 3,000 (Rats 4 and 5, Table 8 and Rats 3, 4, and 5, Table 9) trypanosomes was followed by a seventy-two-hour period of incubation, and the injection of 150 to 300 trypanosomes (Tables 9 and 10) by a four day interval.

(3) Ordinarily, rats heavily injected with this strain of *Trypanosoma brucei* died within three or four days after the intraperitoneal injection of parasites. Infection with smaller numbers lengthened the duration of life; but after the parasites had multiplied to such an extent as to be found in the peripheral blood, the duration of life was about uniform (three to five days), regardless of how many trypanosomes had been injected at the time of infection. In this manner, there was a distinct numeric relation between the number of trypanosomes injected and the time of death in so far that the fewer injected the longer the time required for sufficient multiplication of numbers to fatally intoxicate the host.

CONCLUSIONS

A method is described which has been found simple, fairly accurate, and very useful for transmitting approximately known numbers of trypanosomes to normal rats under sterile conditions and without injury to the seed animal.

By this method, several series of rats have been infected with increasing numbers of *Trypanosoma lewisi*, *T. equiperdum*, and *T. brucei*, and a study made of the minimal number of trypanosomes in each species necessary for infection, as well as the time of true incubation and duration of life. Well-defined quantitative relations were found with *T. brucei*, less with *T. equiperdum*, and least with *T. lewisi*.

The injection of small numbers of pathogenic trypanosomes lengthens the period of incubation and, in this manner, the duration of life, as dating from the time of infection; but when the parasites once appear in the peripheral blood, the duration of life is about the same, regardless of whether the animal was originally infected with a large or a small number.

COCCIDIOSIS IN CATTLE AND CARABAOS *

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In 1912 and 1913, during observations of numerous cases of experimental rinderpest, the great number of "irregular cases" attracted my attention; these had been noted by many observers and invariably classed and accepted as "atypical cases" of rinderpest. Rinderpest had been produced experimentally by the subcutaneous injection of virulent blood into non-immunes.

In August, 1913, I was sent to look after the large number of work animals of the Calamba Sugar Estate at Canlubang, Laguna, where losses from obscure causes among cattle had been severe for some months past.

It is difficult to study a disease or its etiologic factor, if one is unable to recognize it and distinguish it from other afflictions which may, and often do, co-exist. As far as I could remember, no method had been advanced of differentiating rinderpest beyond all question of doubt from other diseases.

Most of the horses and ponies on this estate had perished from surra, but the microscope failed to show the well-known trypanosomes in cattle or carabaos, altho several hundred animals were subjected to the usual blood examinations. Many of the work-oxen were dying and a great number were in such a weak physical condition that they were unable to work. A reasonably careful survey of the situation showed at once that the deaths and condition of the animals were due to different factors. Many of the weak animals, however, suffered from irregular evacuations of the bowels; some had diarrhea, more or less severe, and others had what appeared to be "tropical dysentery." The latter were greatly emaciated and cachectic, but would eat as long as they could. Their temperatures were invariably a degree or two above normal. Others, in somewhat better condition, showed as a common symptom an irregular, persistent diarrhea, with only occasional distinctly dysenteric evacuations. Of about fifty animals that were kept together, some were always neat and trim, fat, and able to do good work, and never sick. Those that had been on the place several years appeared to do best. No particular type appeared to be immune, but the young animals were attacked in greater number than the old. Thirty work-oxen had died during the month of August. In previous months the losses had been, I was told, greater; various causes were ascribed, among others, poisoned weeds and gastritis due to excess of alkali in plants or water.

As soon as possible the sick animals were segregated and divided into different groups so as to facilitate the study, and, if possible, permit an identification of the unknown disease. On August 30, the presence of rinderpest as one of the diseases was established. Clinical history, symptoms, and postmortem findings all sustained the diagnosis.

* Received for publication April 26, 1915.

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Simultaneous inoculations were begun on a lot of thirty-five animals on September 4. These animals were injected with 2 c.c. of virulent blood brought from Alabang, receiving at the same time 100 c.c. of anti-rinderpest serum. Eight of the oxen showed temperatures of 39-40 C. when they were inoculated and one, Ox 303, registered 41.5 C. The animals had been worked hard until the evening before the injections; thus, no opportunity had been given to examine them clinically, except to take one evening and one morning temperature.

The results of these hasty simultaneous inoculations were remarkable. Several of the weak, feverish animals died in a few days; several of the others, after four days, began to have a severe diarrhea, becoming dysenteric. Feces, at first dry, hard, and coated, soon showed large flakes of mucus, tinged with blood. This dysentery was of the same type as that observed in cases of experimental rinderpest during my stay at Alabang, where it was considered pathognomonic. As a result of the rinderpest-blood and anti-rinderpest serum injections, some of the animals were depressed and suffered as if they had ingested corrosive poisons in dilute state. Others rallied after the first few days, but lost strength and drifted into a cachectic condition lasting weeks.

A few days before, while examining blood slides from carabaos for surra, slides of mucus from diarrheal feces were examined microscopically with the result that numerous round or oval, more or less granular, transparent cysts, with a neat double contour were noted, which were at first accepted as ova from intestinal parasites. In the dysenteric feces very few of these could be found, but upon the diaphragm's being closed to the proper point, thousands of elliptic or irregularly oval, granular, transparent bodies, with vague outlines but a well-defined nucleus, were at once observed. These were the same as those observed on slides from the intestines of Bullock 235, which died August 25. On August 26, similar bodies and rounded cysts were found in the feces of Ox 92, an old animal that was greatly emaciated, and afflicted with chronic diarrhea, and occasional dysenteric attacks. The same organisms were found in a middle-aged animal, Ox 74, which died August 27 after a long attack of diarrhea. These oblong, more or less granular bodies were scarce; but other forms, irregularly club-shaped, of indefinite outline, often presenting a well-defined nucleus, were present in great numbers.

Since these organisms were constantly found in immense numbers, and always associated with and usually in the mucous flakes, their relation to the dysenteric feces could not be denied.

On August 28, a group of twelve animals was confined in the Canlubang corral; in the feces of seven of these, the same organisms could be found in great numbers. On September 1, Bullock 37, which had a temperature of 40.4 C. (one degree less than its temperature of two days before), and was very weak and emaciated, was selected as a clinical case of rinderpest and destroyed for postmortem examination. The lesions corresponded to those found in acute rinderpest, except that no ulcers were found in the buccal cavity. Blood taken from the cadaver and injected into a non-immune experimental animal at the Research Laboratory at Alabang failed to reproduce the disease.

It was not until September 20, after making careful observations in the field and in the immunization corrals, and after due considera-

tion of the notes taken at numerous postmortem examinations, that I was able to establish the final diagnosis of acute or chronic coccidian gastro-enteritis.

Once the cause was known, field observations became simplified. During August and September all work-animals suffering from diarrhea or dysentery, as well as those that were cachectic, were sent to the Canlubang corral. Watering troughs for the work-cattle were put up in suitable places and kept filled with artesian water. The corrals were supplied with pumps and troughs, so that even the weak animals could have free access to clean drinking water. This, together with the dividing of the large herds into small units and the feeding of a good allowance of cut forage, decreased the death rate and lessened the number of weak animals.

Forty-four cattle (thirty-four of which were afflicted with the disease in question) which were kept in the Canlubang corral, looked after with reasonable care, fed regularly and supplied with clean creek water in a watering trough, made slow but steady recoveries. Feces from several of these animals, as well as from all those that died, were examined, with invariably the same results. Blood smears were always negative. On October 4, this herd was turned out to graze on a nearby pasture. After they had been driven to grass for two weeks, many began to loose weight, looked depressed, and were languid; several became afflicted with excessive watery diarrhea, but not dysentery. After losing flesh and strength, about one-third of these became anemic, cachectic, and greatly emaciated, and died from coccidian enteritis. Some of them rallied and made a good recovery, but many remained chronic invalids.

It is typical of protozoan diseases that the contagion exists outside the body—in insects (as in malaria or Texas fever) or in water pools or on swampy ground (as in coccidiosis)—and since the virulence of subsequent attacks depends upon the amount of virulent material ingested, these cases were not relapses, but were due to re-infection. This conclusion enabled me to lay the blame on infected swampy pastures or waterholes.

During the time that I was making observations on this herd in Canlubang corral, three different lots of cattle were subjected to simultaneous inoculation against rinderpest. The first lot, containing, as stated, thirty-five animals, was inoculated on September 4; the second lot of thirty was inoculated on September 18; and the third lot of thirty on September 30. The usual method of injecting 5 c.c. of virulent blood from an animal in the early febrile stage of rinderpest, simultaneously with about 300 c.c. of anti-rinderpest serum, was adopted. All the animals that showed, as a result of these subcutaneous injections, fever, and sooner or later diarrhea or dysentery, had in their evacuations blood-tinged mucous flakes, characteristic of coccidian dysentery; clinically they presented symptoms no different from those of the acute type of the field cases studied at the beginning of the outbreak and accepted as acute coccidian enteritis.

Observations were made on more than 120 field cases and 95 animals that were subjected to simultaneous inoculations. Of these, 35 animals were checked up in a systematic manner. Postmortem examinations were made and notes were taken in 32 cases of coccidian enteritis.

Many of the animals that were subjected to simultaneous inoculations made slow and unsatisfactory recoveries; they became anemic, cachectic, and suffered from chronic diarrhea, that would at times assume a more or less dysenteric type; several died and many remained so weak that they were useless as work animals.

The observations on all these inoculation cases of rinderpest, corresponded in every particular with those on the acute or chronic type of the field cases, where the diagnosis of coccidian gastro-enteritis was arrived at after careful consideration of history, symptoms, postmortem reports, microscopic examination, and due consideration of available literature.

The next opportunity that permitted study and verification of the observations taken in the field, came in February, 1914, when I made experiments in the cultivation of rinderpest virus *in vitro*. Part of the immunization sheds at the Pandacan Quarantine Station were reserved to accommodate the animals, while the cultures were kept in incubators and the laboratory work was done in the laboratories of the Biologic Division of the Bureau of Science in Manila.

All of the inoculations and cultures were begun with virulent blood obtained from cases of experimental rinderpest as they had been studied and as they had occurred at the Veterinary Research Laboratory at Alabang, Rizal, P. I.

Two types of animals were used in these experiments: a larger, coarse breed from Dalupiri, all males; and smaller, neater, nervous animals, all females, from the Island of Fuga. From observations made at Alabang, it was known that the Dalupiri animals were more susceptible to one type of disease, and the smaller Fuga cattle to another. This phase of the experiments, if definite observations could be made, would divide the sick animals into two classes.

The first two animals were inoculated on February 9 with 5 c.c. and 2 c.c. of citrated rinderpest blood obtained from Alabang. Both animals, Fuga Female 3717, and the Dalupiri Male, 3696, died on the tenth and twelfth days, respectively, after the inoculation; the diagnosis was confirmed by lesions found at autopsy.

Blood from these two animals was obtained on February 17, when the disease was clinically identified as rinderpest, and mixed, and Fuga Female 3705 and Dalupiri Male 3690 were injected with 10 c.c. of citrated virulent blood each. The Fuga cow succumbed to a clinically not well-marked attack of rinderpest with only moderate temperature. In postmortem examination, however, marked lesions of rinderpest were found.

In Dalupiri Male 3690, a strong, vigorous animal, the disease developed into a well-marked, typical case of acute rinderpest: The highest temperature (41 C.) was observed on the fifth day; the period of high temperature was

from the fourth to the seventh day; on the eighth day the evacuations became offensive, a great deal of mucus being passed. This severe diarrhea, often dysenteric, persisted more or less for twenty days, when the feces began to assume normal form and the animal began to show rapid improvement. On the fifteenth day a dry, scaly skin eruption, extending over neck, shoulders, and back, made its appearance. The type of diarrhea of this animal, and the symptoms as they were produced during the course of this disease, were identical with those of an acute, severe attack of coccidiosis, altho it was caused by the injection of 10 c.c. of citrated virulent blood from clinically identified cases of rinderpest that had shown at subsequent autopsy well-marked typical lesions.

The animal was turned out to pasture on March 17. Since then it has done well, but passes coccidia in feces. On March 28, it was re-injected with 10 c.c. of rinderpest blood, without a reaction; it was immune.

With Dalupiri Male 3690, a clinically typical case of rinderpest with great loss of flesh, marked offensive diarrhea and dysentery, making a slow but steady recovery, we must compare some of the atypical cases, especially those that resulted in death in a few days and showed constipation instead of diarrhea.

Animals that became afflicted in a pronounced manner, in which the clinical symptoms were marked and permitted readily a correct diagnosis, presented no difficulty. In many the reaction was unusually obscure.

The postmortem notes taken during my stay at Calamba had shown that the cause of death, in a certain number of cases, was a septicemia causing hemolysis, with dark, tarry blood which was unable to carry oxygen. Therefore, death invariably resulted in a few days, without great loss of weight—an acute, even per-acute, attack. On the other hand, animals that lived through this first, more or less septicemic period, would invariably become afflicted with a more or less severe diarrhea or dysentery. These lived longer, became gradually weaker, and lost flesh; the pathognomonic symptoms were dysentery, weakness, and slow cachexia, but not acute toxemia as in the first type. Other cases were of the mixed type, the attack being less acute, the symptoms of the hemolytic-septicemic type increasing more slowly and changing gradually into the diarrheal-dysenteric type, finally terminating in death or recovery, as the case might be.

The postmortem findings in the two types of disease presented readily distinguishable features:

The disease of the first, or hemolytic, type produced lesions characteristic of a hemorrhagic septicemia. Discharges from eyes and nose were often present. The blood escaping from cut vessels appeared dark, tarry, did not coagulate well; more or less pronounced hemolysis stained the serum. The vessels permitted escape of tinged serum and cellular elements into the adjacent tissues, thus causing the characteristic hemorrhagic extravasations found throughout the cadaver. In aggravated cases entire regions were found discolored in this man-

ner. Blood-extravasations into the subcutaneous and muscular tissues were frequently observed. The schneiderian membranes were dark red or bluish red, covered with hemorrhages. The mucosa of the larynx and pharynx showed hemorrhagic and croupous inflammation; its epithelium was often eroded. Throughout the thoracic cavity, sub-pleural hemorrhagic extravasations attracted attention at once; often the intercostal spaces, especially on each side of the vertebral column, were stained dark blood color. Hemorrhage or petechial areas were found on the pericardiac sac, on the myocardium, and in the ventricles. The valves of the heart were often deeply stained. The lungs showed, throughout their tissue, more or less marked areas of hemorrhagic infiltration and corresponding discoloration. The lymph-glands were similarly infiltrated. In the abdominal cavity, the serous layers were covered with blood extravasations. The blood vessels appeared dark, ribbon-like, because the fluid escaped through the permeable vessel wall. The mucosa of the fourth stomach was intensely congested, showed numerous hemorrhagic areas and beginning ulcerations in the acute stage. Throughout the intestinal tract, similar lesions were found, all evidently recent and acute, with extensive hemorrhagic extravasations under the mucosa or into the lumen of the bowel. The liver was usually engorged with blood, and sometimes appeared mottled, showing regions of parenchymatous degeneration and biliary stasis.

The disease of the second, or diarrheal, type, on the other hand, invariably presented lesions typical of a severe gastro-intestinal disturbance, due to rapid, more or less extensive destruction of the mucosa by agamic multiplication of coccidia in the different parts of the intestines. If the animal had been sick eight days or more, the cadaver showed marked emaciation. The eyes were clear but sunken. The abomasum, the duodenum, cecum and rectum were usually severely involved, while the lesions in the jejunum, ileum, and colon appeared less marked. The defects of the mucosa permitted escape of blood through the destroyed epithelial layer. Destroyed cells, blood elements, and serum, mixed with intestinal secretions and ingesta, formed the thick, slimy layer covering the mucosa of the affected parts. The mucosa had a more or less offensive odor. It was moreover a favorable medium for the multiplication of secondary (bacterial) invaders. Parts of the bowels involved in this manner could often be recognized as soon as the abdominal cavity was opened; they had a peculiar bluish gray or slaty color, and appeared very anemic. Pleurae, lungs, heart, diaphragm, liver, and mesenteric layers were pale, anemic, but bright and glistening, free from the characteristic hemorrhagic extravasations always found in, and pathognomonic for, the hemorrhagic-septicemic type. The vessels did not permit the blood to escape into the surrounding tissues; the vessels were therefore well defined and distended on a pale, anemic background. The blood was bright red and formed firm, buff-colored clots.

Since to the first type were due the per-acute and acute attacks, and to the second type the somewhat slower acute and chronic cases, it is evident that the diarrheal form supercedes the septicemic-hemorrhagic period. When animals died during certain stages of the pest, the lesions found on autopsy were of a mixed type, approaching more the septicemic or the diarrheal type, according to the type of disease which predominated at the time of death.

If we remember and consider the causes producing the different clinical symptoms and the clinical history, we shall find that in a large number of cases they correspond with the autopsy reports which permit us to recognize as the cause of death: (1) a hemolytic, or septicemic-hemorrhagic, type; (2) a mixed type; and (3) a diarrheal type of disease.

The course of the disease in all animals suffering from the diarrheal type could readily be followed by noting the type of diarrhea or dysentery. The microscope permitted a ready identification of the type and approximate number of coccidia. These examinations could be made in a systematic manner, similar to that of the examination of stools from human sources for bacilli or amebae. Coccidia produced dangerous dysentery only during their asexual multiplication, when young, immature, asexual forms and merozoites were passed out in incredible numbers. When forms produced by sexual multiplication—macrogametocytes, micro-gametocytes, oöcytes, or the smaller cystic forms—began to appear in the mucus, schizogenesis had reached its limit, and the host, if not too weak, would improve to a marked degree in two or three days, and recover.

Such cases could be recognized, studied, diagnosed, and a reasonably correct prognosis made.

If some way could be devised to identify and to diagnose the septicemic cases, enabling us to form a proper diagnosis early, the study of the disease proper could then begin. In an effort to determine what could be done, it appeared that some sero-diagnostic method would be indicated.

Marmorek,¹ in 1909, diagnosed tuberculosis in man by using rabbit and sheep corpuscle hemolytic-amboceptor, urine from tuberculosis patients as antigen, antituberculous serum as antibody, and guinea-pig serum as complement.

This method was tried. In titrating for the complement-deviation values, it was at once noted that the antigen—the urines from animals sick with this type of disease—dissolved the blood corpuscles with marked rapidity, so that it could not be used for this purpose. Since the urine of animals suffering from hemolysis had this property and it was found possible to heat such urines with only slight diminution thereof, the problem assumed a new and more favorable aspect.

After many trials it was found that 0.5 c.c. of a 10 percent emulsion of sheep corpuscle in salt solution and approximately 5 c.c. of fresh but filtered urine gave the best results. Urines can be heated to 60-70 C. for thirty minutes and lose but little of this property. They will then keep for weeks, altho in my cases (about 250 samples) a marked diminution of the clearness of the reaction is to be considered after twenty days.

1. La Presse méd., 1909, 17, p. 12.

A series of suitable test tubes was taken. Into each were placed 0.5 c.c. of 10 percent emulsion of sheep corpuscles; 5 c.c. of the urine to be tested were added, and the tubes were well shaken and set away at room temperature (20 C.) for 24-36 hours. A tube with 0.5 c.c. corpuscle emulsion and 5 c.c. of salt solution was used as one check, and another tube with 0.5 c.c. corpuscle emulsion and non-reacting urine was used as another. The tubes had to be kept in a perpendicular position.

In urine from animals that were not afflicted with the hemolytic type of the disease, the blood corpuscles would settle in a button on the bottom of the tube, similar to the checks, which did it regularly. The urine above might be clear, hazy, or even turbid. On the other hand, in acute cases of the hemolytic type, a very rapid and complete dissolution of the corpuscles took place, and the entire tube became bright red, and perfectly transparent. When 5 c.c. of urine dissolved 0.5 c.c. of 10 percent blood corpuscle emulsion, the hemolytic factor could be expressed as $0.5/5$ equals $1/10$, showing a dangerous attack. Urines could be titrated as to the amount of emulsion of sheep corpuscles that they could dissolve. A factor of $2/10$, in the cases that I examined, invariably signified death in a few hours or the next day. Between these extremes, all degrees of hemolysis could be observed. The button might form and a dark red zone, extending one centimeter above it, would indicate a moderate, not dangerous, attack. On the other hand, the corpuscles might settle into an indistinct, dark-red, shallow area in the cupola of the tube, with a marked hemolytic zone coloring two-thirds of the urine red.

The course of the disease was followed in samples of urine taken every day. The hemolytic periods were subject to marked variations. In artificially produced rinderpest (blood injections), the reaction was severe on the fourth or fifth day, occasionally sooner, maintained itself during three to ten days, and became gradually less, until at last the corpuscles settled rapidly into a firm button whereupon the animal could be considered very resistant against an injection of approximately the same virulence. As long as the condition became worse, the hemolysis in the tube increased; when the hemolysis decreased and a button of definite form appeared, the animal was improving. The urines were gathered in wide-mouthed bottles and filtered directly into the tubes, which were labeled, heated to 60-70 C. for thirty minutes, cooled and stored away or tested as soon as desired. One tube alone would have proved of course whether the process in question was developing in the animal or not; but a series of tubes showed to a nicety the course of the disease.

Observations on about thirty animals, in all stages of the disease, show that when executed carefully and cleanly this method is reliable and practicable. I do not claim that it is a "specific" reaction, but it is a diagnostic method permitting the observation of otherwise invisible phenomena.

The question will no doubt be asked of why rinderpest should be diagnosed, when this paper discusses coccidiosis.

The two diseases are so intimately associated, are so difficult to differentiate clinically, that I hailed with great delight this diagnostic method and thought that, being able to eliminate coccidiosis with the

microscope, and diagnose rinderpest by urine test, I could now really begin the study of either disease.

A black Dalupiri male, 3694, which had been carefully kept among the non-immune experimental animals, was noted as having a slight but persistent diarrhea beginning in an obscure manner about March 15. On April 22, the diarrhea persisting, the animal was isolated. The evening temperature was found to be 39.6 C., and remained above 39 C. in the evening and above 38 C. in the morning for weeks; a dry, scaly skin eruption on the forequarter and neck appeared; at times there was a slight nasal discharge, and soon after the diarrhea became well established, coccidia could be found in the feces. The animal lost a little weight, was always bright, quarrelsome, relished its rations, and at times ate ravenously. On April 21, coccidia in great numbers were found in the feces, including many young asexual, and a few cystic forms. This type of animal is especially susceptible to coccidian gastro-enteritis. It had been noted before however, when animals of this type were used for rinderpest experiments at the Veterinary Research Laboratory, that they had high temperatures, so that the Fuga cattle were preferred, and the Dalupiri animals were discarded. For this reason Dalupiri Male 3694 was a useful addition to the animals "under observation," permitting the study of the correlation of the hemolytic and the diarrheal types of the diseases. This heretofore had been a difficult problem. Before April 15, urine tests were negative. On April 18, hemolysis extended through one-fourth of the tube; on the 20th, through one-half of the tube; on the 24th, the 28th, and the 30th, through one-fourth of the tube. On May 2, the hemolysis extended through one-fourth of the tube; on the 4th, no hemolysis appeared, nor on the 6th; on May 8, hemolysis extended through one-fourth of the tube; on the 11th, through one-fourth; and on the 13th, again no hemolysis appeared.

This slight but persistent reaction was coincident with the finding of coccidia in the feces, extending over many weeks. The animal was fed on good hay, which temporarily lessened the diarrhea. The high temperature, the remarkable appetite, the dry, slightly scaly skin eruption, the irregular, persistent diarrhea, the presence, every few days, of more or less mucus with the different forms of coccidia, the slight but recurring attacks of hemolysis as demonstrated in the urine, were all due to a naturally acquired coccidian infection. It was the first to come under our observation from the earliest stages.

This case of coccidiosis showed all the symptoms, in mild form, that a pronounced case of rinderpest presented; nothing was wanting, the animal having shown in the beginning a slight nasal discharge and a slight discharge from the eyes.

Again, on April 18, a medium-sized carabao, brought in from the country, came under observation. It appeared perfectly normal; the temperature was not markedly increased, but the feces were irregular, at times soft, at times coated with mucus; occasionally appreciable masses (not flakes) of mucus were expelled, and these showed schizogenous forms of coccidia in enormous numbers in every microscopic field. In the thin flakes of mucus, the sexual, cystic forms were numerous. The urine showed no hemolysis on May 18 or May 20; slight hemolysis on the 22d; and hemolysis extending through one-fourth of the tube on the 25th. The animal was then taken away and could not be observed further.

It was decided to subject Dalupiri Male 3694 to a test inoculation of rinderpest blood, because it appeared that, if the diseases were identical, immunity would certainly have been produced between April 22, the date of isolation, and June 11, the date of injection of 5 c.c. of virulent blood. The high virulency of this amount was demonstrated by the severe attack experienced by other animals in the same series.

The temperature of the animal at the time of the injection was 39.4 C. in the evening and 38.3 C. in the morning, indicating a chronic, more or less serious disturbance. On the fourth day after the injection of virulent blood, the temperature rose to 39.5 C. and 40.6 C., and on the fifth day to 40 C. and 40.5 C. Diarrhea became aggravated; dysentery began on the ninth day and rapidly increased in severity; still Dalupiri Male 3694 showed a remarkable resistance and was eating a little grass one day before it died—emaciated to a skeleton.

Postmortem lesions were absolutely identical with those of rinderpest. Scrapings from the ulcerated abomasum, the duodenum, the cecum, the colon, and rectum, however, showed young forms of the schizogenous type of coccidia in countless numbers.

To diagnose rinderpest correctly, and to differentiate it from coccidiosis will require further study and evidently a series of most carefully executed experiments and observations. In the attempts to solve this important problem, another dangerous and serious disease, the ravages of which have caused enormous losses in Africa, was uncovered and identified and the means brought out to establish a correct and positive diagnosis, so that a careful study of coccidian gastro-enteritis, as found in these Islands, can now begin.

Coccidiosis, or, to be more definite, coccidian gastro-enteritis, was observed as early as 1882 on high pastures in some parts of Switzerland. In 1892 Zschokke² and Hess,³ and in 1893 Guillebeau,⁴ published comprehensive articles on the "red dysentery of cattle" caused by the schyzogenous multiplication of coccidia in the epithelial cells of the intestinal canal. All the text-books on veterinary medicine and therapeutics, as well as on protozoan parasitology, refer to these articles. Hess states that, during the year 1900, 200 cases occurred in the district of Saanen, of which sixteen died; that during the year 1891, 180 cases were noted, with a loss of twenty. Most of the cattle made good recoveries in eight days, some in two to three weeks, while other cases lasted two to three months. The animals became greatly emaciated and cachectic. Coccidia could readily be demonstrated in enormous numbers, especially in the abomasum, the cecum, colon, and rectum. Infected drinking water from swampy pastures was established as the cause. Hess quotes four instances in which the infection was traced to hay, the cattle having been kept in stables on dry rations. Züblein's⁵ observations supplement those of earlier writers and he arrives at the same conclusions as to cause and mode of infection. Since then Law,⁶ Degoix,⁷

2. Schweizer Arch. f. Tierheilkunde, 1892.

3. Ibid.

4. Ibid., 1893.

5. Ibid., 1908, 49, p. 123.

6. Veterinary Medicine, 1905, 2 p. 263.

7. Rev. gén. de méd. vet., 1904, 3, p. 177.

Kitt,⁸ Eustace Montgomery,¹¹ A. Balfour,¹² and others have investigated and published articles on this subject.

The organism causing the disease under discussion belongs to the phylum Protozoa, subphylum Plasmodroma, class of Sporozoa (Leukart), subclass Telosporidia (Schaudinn). F. Doeflein¹³ places them as first order Coccidiomorpha (Doeflein), suborder Coccida (Leukart). They are related therefore in some of their characteristics to the Hemosporidia and Gregarina. Their complex life cycle is well described and illustrated, by Doeflein, but the nomenclatures of different authors, as usual, do not agree. It would appear that the coccidia which were observed are not identical with *Coccidium oviforme* (Stiedia eimeria). On three different occasions, oöcytes with eight free, circumposed, round spores were observed. Whether this will be found to be constant, or whether it was an artefact, only a careful observation will demonstrate. Since coccidia are classified by the number of sporozoites formed and their methods of division, this point must be considered as very important in a final classification of these organisms.

The class to which the coccidia belong being known, a close scrutiny of the parasite can be made, because sporozoa as a class come under the same general biologic laws.

They all form oöcytes (sporozoites), and these are the forms that permit the termination of the life-cycle outside the body of the host, and, through the formation of spores, transmit the disease by means of water and forage. Since sporozoites result only from sexual division, the chronic carriers, in which schizogony is limited and therefore not dangerous to the host, are the distributors of the forms that infect the swampy pastures and, multiplying more or less, subject to the stimulus of more or less favorable environment, re-infect new animals and destroy some sensitive new host by their often incredibly rapid asexual (schizogenous) division.

F. Doeflein¹³ makes the statement that many parasites, resembling sporozoites, found free in the blood stream, have been described as *Drepanidia*, whereas they probably were coccidia, in certain stages of development, circulating in the vascular system.

If we take blood from an animal recovering from a severe attack of coccidian dysentery, we can, by careful centrifugation and laking, obtain spores in appreciable numbers. They can also be recovered from feces by sedimentation methods. In urine they are found far more easily, but only the smaller forms, appearing as dense, small coccoid bodies under the oil-immersion, can be obtained. The larger forms are held back, apparently by the kidney.

When spores are first liberated, they are quite large, about one-half the size of an erythrocyte. They take eosin or carbolfuchsin stain, especially when first subjected to a mordant of chromic acid. They lose the color readily however, 50 percent alcohol or 2 percent sulphuric acid decolorizing all the large forms in a few minutes. As the spores become more developed, they show a denser capsule and become smaller. We must also consider that coccidia are readily influenced to modify their method of division; Guillebeau⁹ and Züblein⁵ report that at 20-30 C. the usual division and sporulation takes place in *Coccidium oviforme*, but that at 39 C. the division into very small, spheroid

8. Lehrbuch d. path. Mikroskopie, 1908.

9. Tierärztliche Rundschau, 1914.

10. Ann. rep. vet. path. Lab., Nairobi, British East Africa, 1911-12, p. 10.

11. Bull. Soc. path. exotique, 1910, 3, p. 293.

12. Ibid., p. 429.

13. Protozoen-kunde, 1911.

bodies is most rapid, and reaches a point where the resulting spheroidal bodies cannot be seen by high power.

In regard to occurrence, Doflein¹⁴ states that coccidia are distributed over the entire world. In these islands they can probably be found in all animals, wherever mucous flakes can be detected, by a careful microscopic examination of the dejecta. During the last dry season, the animals on a certain tract of land on the Pasig River showed normal feces. About two or three weeks after the early heavy showers, all the immune cattle, Chinese and Indian, as well as sheep, goats, horses, and later, even hogs, showed marked constipation, the feces being passed in hard, formed masses, often hanging together on mucous strings; in these flakes the sexual division forms of coccidia were found in great numbers.

A herd of twenty-one carabaos, fine large animals, imported a few years ago from Pnom-Penh, apparently in the best of health, were all constipated, the feces appearing coated, or varnished. Examinations under the microscope always established the same diagnosis. Cattle from Mindoro were also markedly affected. Cattle from the Batanes Islands, especially a shipment of carabaos and cattle from Dalupiri, were greatly constipated, their feces formed into small, hard, dense pellets, covered with blood-tinged mucus, which could be recognized with the naked eye. One of this lot, however, an old cow, had a marked attack of diarrhea, during which masses of mucus with the well known schizogenous forms appeared. This early constipated stage is characteristic of coccidiosis and is superseded by the dangerous dysentery only if the host is susceptible and his body cells cannot inhibit the rapid and destructive agamic multiplication.

A notable exception is to be recorded in chickens, which show the symptoms early in the coccidian season, usually by a muco-sanguineous diarrhea with large intestinal mucous casts, in which the club-shaped merozoites alone appear, often in rosette formation, not yet separated. The birds stop laying, are dumpish, their combs bluish or gray; they appear as if they were going through a heavy molt, without losing their feathers.

It appears therefore that coccidiosis, altho it has escaped notice so far, has an extensive distribution throughout the Philippine Islands.

While carriers of chronic coccidia are not seriously affected, having acquired immunity themselves, they infect the feeding grounds and watering places of susceptible animals. More information on this phase of the coccidial life-cycle must be obtained. We do not know the exciting cause, the stimulating factor, that produces the epidemic outbreaks among cattle. From the numerous observations made in our rinderpest experiments, it would appear, in view of the peculiar methods by which coccidia can be divided into most minute organisms (Züblein, 1908), that one part of their life-cycle is passed in the blood vascular system, and that their rapid multiplication in most minute forms produces the hemolytic form of the disease. This is superseded, if the host lives, by the diarrheal form, the latter being necessary to

14. Centralbl. f. Bacteriol. and Parasitenkunde d. Infek. Krankh., 1911, 50, p. 7.

re-infect other hosts and to transfer the contagion from one place to another.

Guillebeau³ (1893) states that some animals succumb on the second day. This is impossible even if the epithelial cells are damaged by excessively rapid schizogenesis. The rapid multiplication in the blood stream of an organism which is ultra-microscopic during a part of its life-cycle, offers the best and most plausible explanation. Loeffler¹⁴ (1911) states that the different results obtained by various investigators relating to the filtrability of the virus of a certain disease may often be due to the fact that the virus from the same strain is composed of larger or smaller particles, according to the stage of development at which it happens to be in the material that is filtered.

E. Montgomery,^{10,11} in speaking of an epidemic outbreak of coccidiosis in East Africa, states that, clinically, coccidian gastro-enteritis cannot be differentiated from rinderpest, but that rinderpest can be transmitted by blood-inoculations, while this is not the case with coccidiosis.

My observations show that the history, development, clinical symptoms, and postmortem findings of acute coccidian gastro-enteritis and acute rinderpest cannot be distinguished, that they are identical, as Montgomery states. In one disease, however, coccidia in enormous numbers can always be demonstrated in intestinal scrapings and slime from the abomasum, duodenum, cecum, colon, or rectum, obtainable in large quantities on postmortem examinations.

We cannot deny that acute coccidiosis has invariably resulted from rinderpest inoculations. Our last cases (3,694 and 3,714) died from coccidian gastro-enteritis of the most severe type after receiving a subcutaneous injection of 5 c.c. of rinderpest blood on June 11. Since we have been able to recognize coccidiosis, we have never found a case of rinderpest without the presence of these organisms in great numbers.

Animals immune to rinderpest have, in the large majority of cases, been observed to be periodic carriers, or secretors, of coccidia in different forms. As examples, may be cited the immunized work-oxen (Chinese) and the Nellore Indian cattle from the Iwahig Penal Colony, on the island of Palawan, used as work animals by the Bureau of Agriculture. Of four guinea-pigs that were inoculated on April 21 with blood from spore-carrying cattle, two died about June 12 from coccidian dysentery, one died from pneumonia, and one is still alive and doing well. Dr. E. L. Walker's guinea-pigs, kept in the same room and fed in the same way, have never shown any disturbance; Dr. E. H. Ruediger, a careful observer, has assured me that none of his small animals have died from similar trouble during this period.

Many authorities have arrived at the conclusion also that rinderpest cannot be a bacterial disease, that it belongs to the scourges due to protozoan invasion. Dr. Youngberg states that he has observed animals that had been immunized which showed more or less diarrhea. The peculiar behavior of animals susceptible to rinderpest indicates that they have only a lesser or greater resistance. The numerous "relapses" observed in the Calamba outbreak were nothing other than re-infections due to infected pastures. This does not correspond with bacterial immunity, but finds a ready explanation when the general laws underlying the acquirements of immunity, or to be exact, resistance against protozoan diseases, are considered.

A good illustration of nature's method of producing immunity against protozoa is found in our southern states, where cattle, inoculated by ticks with piroplasmiasis for generations, have acquired a very high resistance. Again, in the Tropics, malaria is the cause of many deaths among children,¹⁵ while it finds adults very resistant, altho often carriers and susceptible when injected with large doses of infected blood.

Mrowka,¹⁶ who had ample opportunity to observe the East-Asiatic rinderpest, states:

No doubt can exist that the virus is found in the body in latent form, that the cause for relapse is to be looked for in the mucosa of the abomasum, and that its virulency is re-established by excessive exertions or changed conditions of life which affect the immune animals unfavorably.

This explains why animals, able to resist artificial and natural infection, and, therefore, apparently immune, occasionally become sick with rinderpest, so that the words "immune to rinderpest" really mean afflicted with rinderpest in latent form.

We must distinguish virus carriers and virus secretors. All animals immune to rinderpest, though absolutely harmless to their surroundings, may have a relapse and thus become virus secretors. This peculiarity identifies rinderpest as one of the many protozoan diseases.

15. Deutsch. med. Wchnschr., 1900.

16. Ztschr. f. Infek. Krankheiten d. Haustiere 1913, 15, p. 139.

THE "REACTION" OF BACTERIOLOGIC CULTURE MEDIA *

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INTRODUCTION

It would be a large task to compile all the recorded instances in which the reaction of a medium has influenced the morphology, pigment formation, proteolytic activity, rate of fermentation, or even the mere viability of bacteria. In 1895 Fuller¹ had collected "over a hundred" such cases; to-day they occur so frequently in the literature that there is no need to emphasize the importance of the subject.

Adjustments in the reaction of bacteriologic culture media are generally accomplished by titrations. General use of such methods dates from the suggestions made by Schultz² (1891), Fuller¹ (1895) and others in the early nineties. Founded upon these methods are the "scales of reaction." They are eminently useful, and, now that a vast deal of laboriously-gained experience has dictated in terms of these scales the proper reaction of important media, they are not likely to be easily displaced.

But the theory of titration has undergone a fundamental change since the origination of these methods, and we are now able to gain a clearer conception of many points which were of necessity inadequately treated by the early writers. Furthermore, the physiologic importance of "titratable acidity" is dwindling before the broader conceptions of modern physiologic and physical chemistry, and it may be seriously suggested that too strong an adherence to the old titration methods is a hindrance to progress.

It has long been recognized that acidity and alkalinity are best defined in terms of hydrogen ion concentration. Such a conception certainly correlates a vast number of facts. The rate of enzyme action, the stability of colloidal structures upon which cellular life depends, the solubility of many physiologically important compounds,

* Received for publication May 3, 1915. Published by permission of the Secretary of Agriculture.

1. Jour. Am. Pub. Health Assn., 1895, 20, p. 381.

2. Centralbl. f. Bacteriol., I, O., 1891, 10, p. 52.

as well as the structure and composition of media and the color of the indicators used in the adjustment of their reactions—all are dependent in greater or less measure upon hydrogen ion concentrations.

This is doubtless very generally recognized; but no attempt has been made, so far as the writer knows, to apply these principles extensively to bacteriologic culture media.

Hildebrand,³ in assembling some of the principles of titration for use by chemists, has suggested that the application of such principles may have been delayed because the apparatus or the mathematics involved have appeared too complicated for men not trained in theoretical chemistry. Some of the difficulties involved in older methods may be avoided by the use of the hydrogen electrode. With this instrument we may determine directly hydrogen ion concentrations. Important points in the data so obtained may then be presented by graphic methods quite as well as by equations. In order to make these presentations clear, it will be necessary to sketch certain principles and to deal with certain questions which already have been set forth more adequately by others. To Hildebrand's³ paper and to Michaelis'⁴ monograph, the writer is particularly indebted.

METHODS

It may be well to recall that the customary method of determining hydrogen ion concentrations electrometrically depends upon a measurement of the potential of the chain:

Hg HgCl/n/10 KCl/saturated KCl/solution/PtH₂, for which we have at 30 C. the equation $E - 0.337 = 0.0601 \log 1/CH^+$, in which E is the total E. M. F. of the chain, 0.337 is the potential of the "tenth normal" calomel electrode, in terms of "the normal hydrogen electrode," and CH⁺ is the hydrogen ion concentration of the solution. This equation may be transposed to $E - 0.337/0.0601 = \log 1/CH^+$. The value of $\log 1/CH^+$ or $-\log CH^+$ is obtained directly, and since it is a much more convenient value to plot than the unwieldy value of the corresponding hydrogen ion concentration, it is used under the symbol pH⁺ introduced by Sørensen.⁵

In the experiments to be described later, this chain was used at 30 C. The electrode vessel was a modification of Hasselbalch's⁶ and will be described in detail elsewhere. It was found that the contact potential between the n/10 KCl and the solution was reduced to a minimum by the use of the customary inter-

3. Jour. Am. Chem. Soc., 1913, 35, p. 847.

4. Das Wasserstoffionen-Konzentration, 1914.

5. Compt. rend. du Lab. Carlsberg, 1909, 8, p. 1.

6. Biochem. Ztschr., 1913, 49, p. 451.

posed solution of saturated KCl. The design of the apparatus was such that this could be accomplished especially well, and since the corrections by Bjerrum's⁷ method of extrapolation would be small and would have little effect upon relative values, they were neglected.

A small correction for barometric pressure and sometimes a correction of the calomel electrode, found by comparison with other assumed standards, were applied.

The electrodes were platinized platinum or gold coated with palladium black. The two types agree excellently, and no electrode was used which differed from the five comparison electrodes by more than 0.0001 volt after a night in the same solution. In the great majority of measurements, the electrodes used had agreed within $\pm 0.000,030$ volt before they were transferred to the electrode vessel.

All solutions were brought to $30\text{ C.} \pm 0.1^\circ$ in a water bath and then, before measurement, their containers were wiped dry and they were left about half an hour in the air thermostat containing the hydrogen electrode. This thermostat was kept constant to within $\pm 0.05\text{ C.}$ (Beckmann thermometer reading) by a special regulator.⁸ Aside from a slow drift in temperature, the fluctuations from moment to moment were less than $\pm 0.003^\circ$, determined by an eight-pair copper constantine thermo element.

Measurements of potential were made with a Leeds and Northrup potentiometer and a galvanometer as null point instrument. The potentiometer was calibrated by the Bureau of Standards. The known potential was derived from a Weston cell, frequently compared with two Weston cells which had been standardized and loaned by the Bureau of Standards.

The sodium hydroxid used in titrations was practically carbonate free. It was made from carefully cleaned Kahlbaum sodium held in a silver crucible. The crucible was placed in a desiccator containing a layer of distilled water and held there until the sodium was completely converted to NaOH. From this the standard solution was made up. It was held in a paraffined Jena flask with protecting soda lime tubes and was delivered to a measuring burette through a glass tube sealed to the burette. The standardization was made by Morey's⁹ benzoic acid method.

In cases in which lactic acid was used in titrations, the acid was boiled in dilute solution to convert the anhydrid to acid.

In stating the composition of media, the percentages of peptone, gelatin, etc., are not based on dry weights, since the object was to present data on media as ordinarily made up. Attention should also be drawn to the fact that a "1 percent peptone" solution is a solution made by treating 1 gm. Witte peptone with 100 c.c. water, heating, filtering, and making up to volume. By no means all the peptone goes into solution.

METHOD OF PRESENTING DATA

In presenting results, use is to be made of titration curves and it is essential that these should be understood. As an illustration, let us borrow an example given by Böttjer¹⁰ and reproduced by Hilde-

7. Ztschr. f. Electrochem., 1911, 17, p. 589.

8. Jour. Am. Chem. Soc., 1913, 35, p. 1889.

9. U. S. Dept. Commerce and Labor, Bureau of Standards, 1913, Bull. 8, p. 643.

10. Ztschr. f. physik. Chem., 1897, 24, p. 253.

brand³ and Bjerrum.¹¹ In Chart 1 are sketched the titration curves of hydrochloric and acetic acids.

Let us start with 10 c.c. of $n/10$ HCl. Approximately 90 percent of the acid is dissociated. Hence, the normality of hydrogen ions in this 0.1-N HCl solution is about 0.09, written $CH^+ = 9.0 \times 10^{-2}$. If to this solution successive portions of $n/10$ NaOH are added, the acid becomes neutralized and the hydrogen ion concentration declines. After each successive addition of NaOH, we may measure the hydrogen ion concentration with a hydrogen electrode. In this measurement we obtain directly, as shown under Methods, the value of $\log 1/CH^+$, which, under the symbol pH^+ , we shall use in plotting the results. It will be noticed that pH^+ increases as CH^+ decreases. All the charts in this paper have been constructed to show a rise in the curves with a rise of hydrogen ion concentration, and conversely, a fall in the curves with a decline in hydrogen ion concentration. On the abscissae of Chart 1, will be found the cubic centimeters of $n/10$ NaOH added to each 10 c.c. of titrated solution.

Plotting the results during a titration of HCl with NaOH, we obtain the curve shown in Chart 1. Each addition of NaOH causes a fall in hydrogen ion concentration, but it is not until almost all the acid has been neutralized that a sudden fall in the curve appears. When 99.9 percent of the acid has been neutralized, we should still have a 0.0001 N. HCl solution which, diluted with the water of the NaOH solution added, has become approximately 0.00005 N. HCl. Neglecting the influence of the NaCl and assuming that the acid is completely dissociated, we should then have a hydrogen ion concentration of approximately 5×10^{-5} . The next 0.07 c.c. of alkali added causes a precipitous drop in the hydrogen ion concentration, and, as the point of complete neutralization is passed, the curve falls rapidly to the region where we find the hydrogen ion concentrations of the sodium hydroxid.

It will be remembered of course that, according to the fundamental equation, $\text{Concentration } H^+ \times \text{Concentration } OH^- = \text{a constant}$, we must have hydrogen ions even in the presence of an excess of hydroxyl ions. Therefore, the alkalinity of solutions may still be defined in terms of the concentration of the hydrogen ions.

Turning again to Chart 1, we find there the "titration curve" of a weak acid, acetic. We proceed as before, and we notice in the first

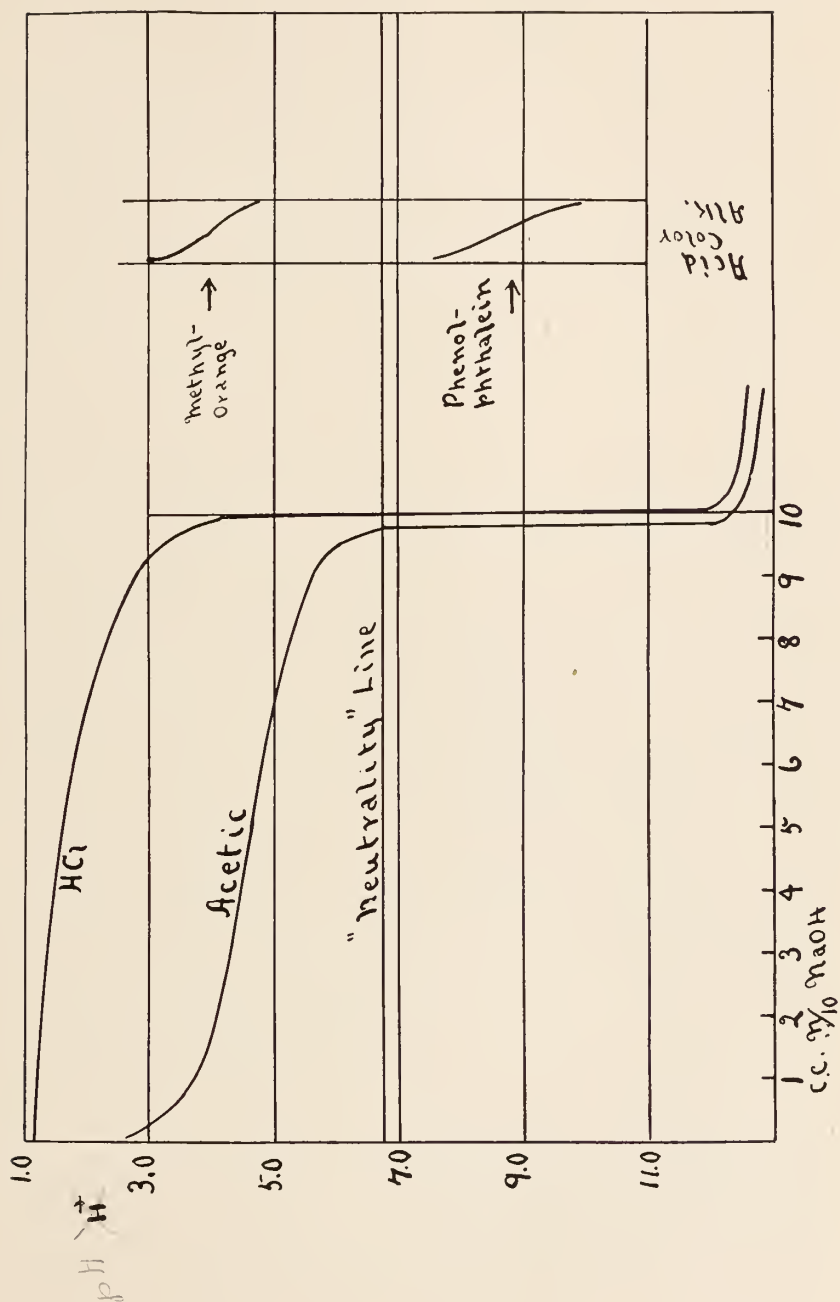


Chart 1.—Titration curves of hydrochloric and acetic acids.

place that the initial hydrogen ion concentration is much lower than that of the corresponding hydrochloric acid solution. In other words, acetic acid is weakly dissociated. Furthermore, the formation of sodium acetate upon the first addition of NaOH depresses the hydrogen ion concentration. Then the curve falls slowly until, as complete neutralization of the acid is approached, we have again the precipitous drop in the curve. All the while, however, the hydrolysis of the sodium acetate, as well as the effect of the acetate ion upon the dissociation of HAc, has made itself felt; and so, before complete neutralization is reached, the drop in the curve takes place. When the acid and alkali are present in equivalent concentration, the solution is distinctly alkaline, and we have, instead of a neutral salt, as in the case of NaCl, a hydrolyzed salt of a weak acid with a strong base.

USE OF INDICATORS IN TITRATION

We may illustrate the usefulness of indicators in titrations by means of the titration curves just given. Without discussing the dissociation, tautomeral changes, or, in some cases, the colloidal properties of indicators, we may define them as substances which undergo changes in color within certain zones of hydrogen ion concentration. Through the work of Fredenthal,¹² Fels,¹³ Salessky,¹⁴ Salm,¹⁵ Sørensen,^{5, 16} Palitzsch,¹⁷ Michaelis and Rona,¹⁸ Walpole,^{19, 20} and others, we now know the range of color changes of a large number of indicators. Phenolphthalein, for instance, to the unaided eye changes gradually from colorless to red between about $\text{CH}^+ = 1 \times 10^{-8}$ and 1×10^{-10} , while methyl orange undergoes its color change between about $\text{CH}^+ = 1 \times 10^{-3}$ and 4×10^{-5} .

Particular emphasis, as we shall see later, must be placed upon the fact that the color change occurs in a zone of appreciable width.

An attempt has been made to present graphically the zone of color change. The degree of color of phenolphthalein at any point between $\text{pH}^+ = 8.00$ and $\text{pH}^+ = 10.00$ is shown with an accuracy sufficient for present purposes by the curve of color change shown at the right in Chart 1, and reproduced in the other charts. In the same way, the

12. Ztschr. f. Electrochem., 1904, 10, p. 113.

13. Ibid., p. 208.

14. Ibid., p. 204.

15. Ztschr. f. physik. Chem., 1906, 57, p. 471.

16. Biochem. Ztschr., 1910, 24, p. 381.

17. Ibid., 1911, 37, p. 131.

18. Ztschr. f. Electrochem., 1908, 14, p. 251.

19. Biochem. Jour., 1913, 7, p. 260.

20. Ibid., 1914, 8, p. 628.

curve of color change of methyl orange illustrates the passage of the color of this indicator from its full acid color over into its alkaline color as the hydrogen ion concentration falls from $\text{pH}^+ = 3.0$ to $\text{pH}^+ = 4.7$. The zones of color changes of these two indicators are actually much wider than the limits assigned here, but for all practicable purposes the limits assigned will do, since the zones given are those within which the eye detects differences in the color.

Turning again to the titration curve for HCl, we find that when the zone is reached in which methyl orange changes color, the curve has begun to drop, and before we are out of this zone, the curve is falling precipitously. When, therefore, practically every trace of HCl has been neutralized, methyl orange, if present, would show its full alkaline color. Furthermore, a minute addition of NaOH, not only sends the curve through the zone of methyl orange, but straight through a zone far below, in which phenolphthalein changes color. Consequently, both methyl orange and phenolphthalein will display a sudden color change in such a titration and we should describe both indicators as "sensitive."

On the other hand, if we should attempt to use methyl orange in titrating acetic acid, we should find no sudden sharp color change, or "end point." This is simply due to the fact that in titrating acetic acid, as the curve shows, the hydrogen ion concentration passes gradually through the zone of color change of methyl orange. If we wish to observe a sharp color change, we must seek an indicator whose transformation occurs in a region where the titration curve falls precipitously. It will be seen that an indicator suitable to the titration of acetic acid is phenolphthalein.

It does not always follow that a precipitous drop in the titration curve occurs when acid and alkali are present in equivalent amounts. This is shown to some extent by the acetic acid curve. Much more complicated situations arise in the titration of "very weak" acids and their mixtures. An exposition of the principles applicable under these circumstances is given by Bjerrum.¹¹

In the titration of most media, containing as they do phosphate and various salts, protein, and amino-acids, often of unknown structure and generally in unknown relative proportions, it is impossible to decide to what point the mixture must be titrated in order to neutralize the acid groups. When such a mixture is titrated, the only thing accomplished is to reach some more or less definite hydrogen ion concentration as determined by the color of an indicator.

"BUFFER" EFFECT

There is one more point which we may illustrate by the curve for acetic acid. When we reach the region where both acetic acid and sodium acetate are present in appreciable quantities, we have a solution the hydrogen ion concentration of which is not easily altered by addition of either acid or alkali. This is expressed graphically in the flatness of the curve. If we add acid to an acetic acid-sodium acetate mixture, a portion of the sodium may be roughly described as parting company with the acetate ion to form a new salt, leaving the acetate ion to become weakly dissociated acetic acid. Largely because of this "weakness" in the dissociation of acetic acid, the hydrogen ion concentration of the mixture is not greatly affected. So, too, the addition of a small amount of alkali does not greatly depress the hydrogen ion concentration, since the alkali finds plenty of undissociated acetic acid with which to neutralize itself.

This is a very rough sketch of an example of what is known as "buffer" action. Any substance which tends to preserve the original hydrogen ion concentration of its solution upon the addition of an acid or a base, is called a "buffer" or "regulator."

TITRATION CURVES OF CULTURE MEDIA

In Charts 2-6 are shown the titration curves of some important media. In these figures the abscissae represent the amount of acid or alkali added (acid to the right, alkali to the left of the 0 ordinate). On the ordinate are to be found the pH^+ values. As a reference line, that of $\text{pH}^+ = 6.86$ is drawn to represent the hydrogen ion concentration of pure water at 30 C. This point is "absolute neutrality." The point $\text{pH}^+ = 8.50$ is assumed to be the "end point" of phenolphthalein, an arbitrary assumption, which will have to be used in later discussions. Accordingly, the $\text{pH}^+ = 8.50$ line has been made heavy to serve as another reference line.

To determine the values from which these titration curves were constructed, the following procedure was followed:

Aliquot samples of a medium were apportioned in clean Jena Erlenmeyer flasks. To each was added a known amount of $n/10$ acid or alkali. These flasks were suspended in a water bath at 30 C. for about one-half hour; then having been wiped dry, they were placed in the 30 C. thermostat containing the hydrogen electrode. They were left there for another half hour. Then the determinations were made on each sample with care that, before each determination, the electrode vessel should have been thoroughly rinsed with a portion of the sample to be tested.

A study of the data so obtained reveals many interesting facts, which will be considered under separate headings.

REACTION OF CULTURE MEDIA TO PHENOLPHTHALEIN

Phenolphthalein is the indicator most commonly used in the titration of culture media. It is important therefore to note certain difficulties which attend its use.

It has already been pointed out that phenolphthalein, like all indicators, does not change color suddenly upon passing a certain hydrogen ion concentration. The zone of color change is broad, and it is only when a titration curve falls precipitously through this zone that the indicator flashes from one color to another.

With each of the titration curves (Charts 2-6), there will be found a graphic representation of the color change of phenolphthalein. This method of representation has been explained under Methods of Presenting Data. It readily will be seen that no sharp color change is to be expected in the titration of any of the media. In other words, none of the titration curves falls precipitously through the zone of phenolphthalein color change. Therefore, there can be no sharp end point in such a titration. To show the variation which arises from the assumption of different tints as end points, a 1 percent peptone solution and a 5 percent peptone solution were submitted to four bacteriologists and four chemists for titration. In each case the observer used the same calibrated burette and pipette, the same $n/40$ NaOH solution, the same phenolphthalein solution, the same carefully rinsed flasks, the same white background. Each observer, however, was asked to proceed as he was accustomed to do. The following results were obtained.

TABLE 1
TITRATION OF PEPTONE SOLUTIONS

Observer	Amt. in c.c. of $n/40$ Alkali Required to Each 5 c.c. of	
	1 Percent Peptone	5 Percent Peptone
Bacteriologist A.....	0.76	3.35
Bacteriologist B.....	0.80	3.25
Bacteriologist C.....	1.40	5.10
Bacteriologist D.....	1.00	4.45
Chemist E.....	0.58	2.68
Chemist F.....	1.12	7.40 (heated)
Chemist G.....	0.70	4.20
Chemist H.....	0.83	4.38

These wide differences are due, in part, to the variations in procedure and appear large because of the dilute alkali used; but for the

most part the differences are attributable to the assumption of various tints as the end point. This will become strikingly evident if the reader will transpose the titers given above to the titration curves of the 1 percent and 5 percent peptone solutions. The locations will be found to fall along the curves within almost the entire range of color change of phenolphthalein.

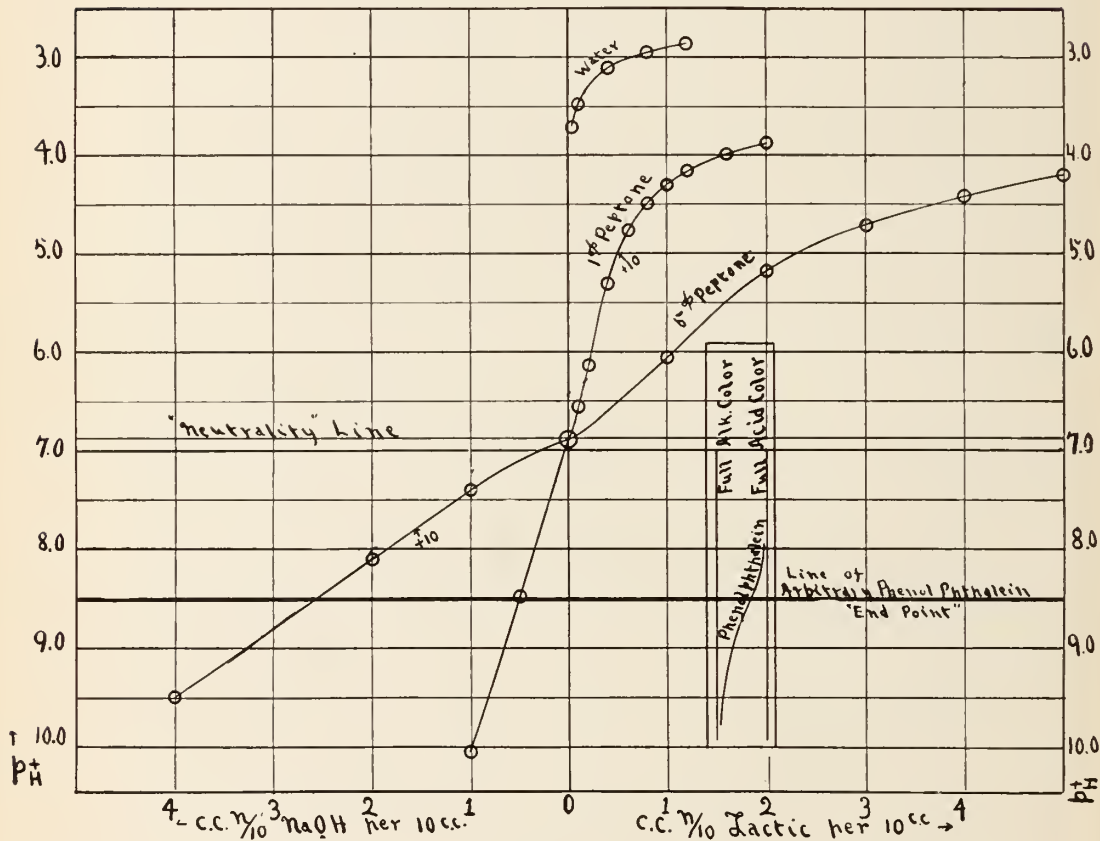


Chart 2.—Titration curves of 1 percent and 5 percent solutions of Witte peptone.

This recalls the well-known necessity for fixing some arbitrary tint as standard. Such a standard is defined by the American Public Health Association²¹ as follows: "A faint but distinct pink color marks the true end point. This distinct pink color may be more precisely

21. Standard Methods for the Examination of Water and Sewage, 1912.

described as a combination of 25 percent red (wave length approximately 658) with 75 percent white, as shown by the disks of the color top,”

There are several objections to such a standard. In the first place, as Ridgeway²² has emphasized, it is extremely difficult to reproduce lithographically a particular color. Furthermore, when produced, it fades upon exposure. Perhaps these objections would be of small consequence if comparison paper colors could be conveniently used. They certainly can not be so used. The most serious objection to such a standard is that no precise physical significance is attached to it, and without such a significance the standard may justly be ignored and is ignored.

A standard with an exact physical significance for the problem at hand would be a solution of known hydrogen ion concentration containing a definite amount of phenolphthalein. Solutions with well-defined hydrogen ion concentration may be accurately reproduced from acetic acid-sodium acetate mixtures,²³ phosphate mixtures,²⁴ and borate mixtures,⁵ as well as from a variety of other mixtures.

In passing, it may again be emphasized that the titrimetric method has for its basis the color change of an indicator within a zone of hydrogen ion concentration, and that the sharpness with which the color change appears is dependent upon the slope of the titration curve within this zone of color change. It follows that, when the end point is sharp, the slightest error in titration makes an enormous difference in the hydrogen ion concentration of the solution. A medium in which a sharp end point is found, is therefore, in general, not desirable. Such media have been automatically eliminated from general use.

In the following discussion, there will be occasion to assume a particular point on the titration curve as the end point of phenolphthalein. Since at $\text{pH}^+ = 8.50$ phenolphthalein is sufficiently pink to be distinctly noticeable in colored media, we shall assume this to be the so-called end point.

There is a further assumption involved; that is, that the indications of phenolphthalein in protein and salt solutions are comparable. There is good reason to believe that within certain narrow limits they are. This subject will be studied in detail in its relation to media and reported in due time.

22. Color Standards and Color Nomenclature, 1912.

23. Walpole, Jour. Chem. Soc., 1914, 105-106, p. 2501.

24. Prideaux, Biochem. Jour., 1911, 6, p. 122.

THE BUFFER EFFECT OF MEDIA

The most important point shown by the titration curves is the buffer effect of the various media. The flatter the curve the greater is the buffer effect of the solution. This is shown clearly by a comparison of the titration curves of the 1 percent and the 5 percent peptone solutions (Chart 2). Both peptone solutions are initially very close to the point of absolute neutrality. On the addition of either acid or alkali, the hydrogen ion concentration of the 5 percent peptone solution changes much less than that of the 1 percent peptone solution, because of the higher content of substances which act as buffers (see under "Buffer" Effects). To bring the 5 percent peptone solution down to $\text{pH}^+ = 8.50$ requires 2.5 c.c. $n/10$ NaOH to each 10 c.c., while to bring the 1 percent peptone solution to the same point requires only 0.5 c.c. $n/10$ NaOH to each 10 c.c. If $\text{pH}^+ = 8.50$ were assumed as the end point with phenolphthalein, the two solutions would have the "reactions" +25 and +5, Fuller's scale.¹ If, now, we should "correct" these solutions each to +10 Fuller's scale, the one would require the addition of $25 - 10 = 15$ c.c. normal NaOH to each 1000 c.c., while the other would require the addition of 5 c.c. *N acid* to each 1000 c.c. After making these corrections, we should find the pH^+ value of the 5 percent peptone solution to be 7.80, while that of the 1 percent peptone solution would be 5.00.

These two solutions, then, both practically at absolute neutrality to begin with, have been "corrected" to the same "reaction;" yet the hydrogen ion concentration of the one has been left at a very favorable point for bacterial action, while the hydrogen ion concentration of the other has been raised almost to the limit of growth of *Bacillus coli*, and certainly far above the limit for numerous other organisms.

There occur in the literature some very striking instances of serious errors caused by neglect of such considerations.

It is true however that Dunham's solution, which is practically identical with the 1 percent peptone solution, is seldom or never "corrected." In text books are to be found elaborate directions for the "correction" of "reactions" and emphasis on its importance. Yet there is a significant absence of any direction for correcting Dunham's solution, or sometimes the specific direction that it should not be "adjusted." Experience seems to have dictated what theory could have predicted.

The comparison of the 1 percent and 5 percent peptone solutions furnishes what is perhaps an extreme case of the futility of titration methods of adjusting reactions. Cases less extreme, but of greater

practical importance, are to be found in the comparison of media made with beef infusions and meat extract. In Chart 3 are the titration curves of such media, all containing 1 percent Witte peptone. One contains 0.3 percent Liebig's extract and another contains 0.5 percent Liebig's extract. The "0 C. infusion" medium was made up according to the directions of the American Public Health Association.²¹ The

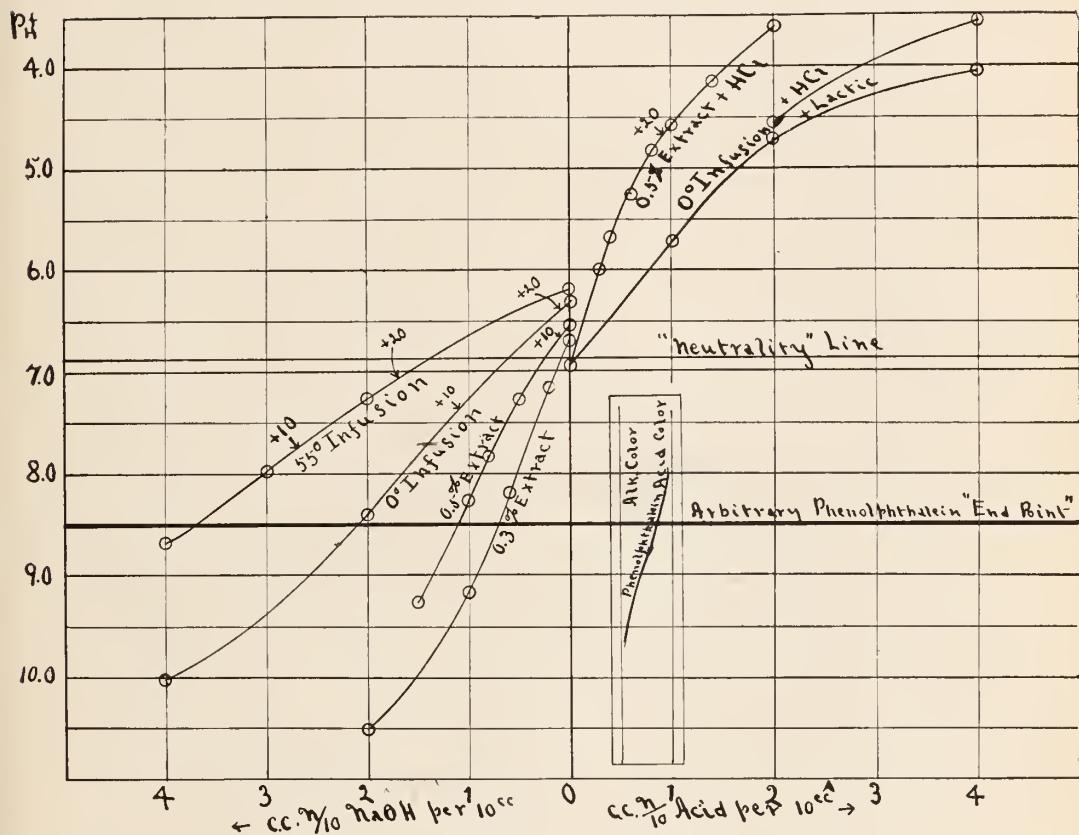


Chart 3.—Titration curves of infusion and meat extract broths.

"55 C. infusion" medium was made up according to Smith.²⁵ The titrations with NaOH were made before sterilization.

One of the most striking facts shown by these curves is the difference in the buffer content of the beef infusion made at 0 C. and that

25. Bacteria in Relation to Plant Diseases, 1905.

made at 55 C. This recalls the work of Gage and Adams,²⁶ who noted remarkable variation in the composition of infusions.

Considering $\text{pH}^+ = 8.50$ as the end point with phenolphthalein and reckoning from where the curves cross the 8.50 line, we point off the +10 and +20 points (Fuller's scale). The location of these points shows again the futility of titration methods of adjusting reactions.

To show the comparative buffer effects of the 0 C. infusion and the 0.5 percent "extract" media when titrated with acid, the two media were brought to the same point, approximately neutral, and titrated with $n/10$ HCl and $n/10$ lactic acid. The difference in these two media in their "buffering" of added acid is of profound importance when such media are used to test the acid fermentation of sugars. If the same organism stops activity at approximately the same hydrogen ion concentration in each medium, the titratable acidity will vary considerably. Consequently, the titratable acidity furnishes no constant. It will vary, as Miss Broadhurst²⁷ has shown that it does, with the composition of the medium. Unless the composition is identical in every laboratory, no comparable data are obtainable. Comparable data are obtainable on another basis, as will be shown in other papers.

TITRATION CURVES OF MEDIA CONTAINING ADDED PHOSPHATES

In view of the fact that phosphates are extensively used in culture media, it may be useful to study a set of curves showing the effect of different phosphate mixtures upon a 1 percent peptone solution. Such curves are shown in Chart 4. These solutions were all titrated with lactic acid to give an approximate idea of the course which the hydrogen ion concentration would run during an acid fermentation of added sugar. In another paper, it will be shown that *Bacillus coli*, in accordance with the observations of Michaelis and Marcora,⁴ brings these media up to approximately the same hydrogen ion concentration. A constant is thereby furnished. Titrations, on the other hand, would furnish a confused mass of data impossible to correlate on the old basis.

TITRATION CURVE OF MILK

It may be interesting to note also the titration curve of milk. In Chart 5 are given the titration curves of two samples of fresh, whole milk, titrated with NaOH, and of two other samples, titrated with lactic

26. Jour. Infect. Dis., 1904, 1, p. 358.

27. Jour. Infect. Dis., 1913, 13, p. 404.

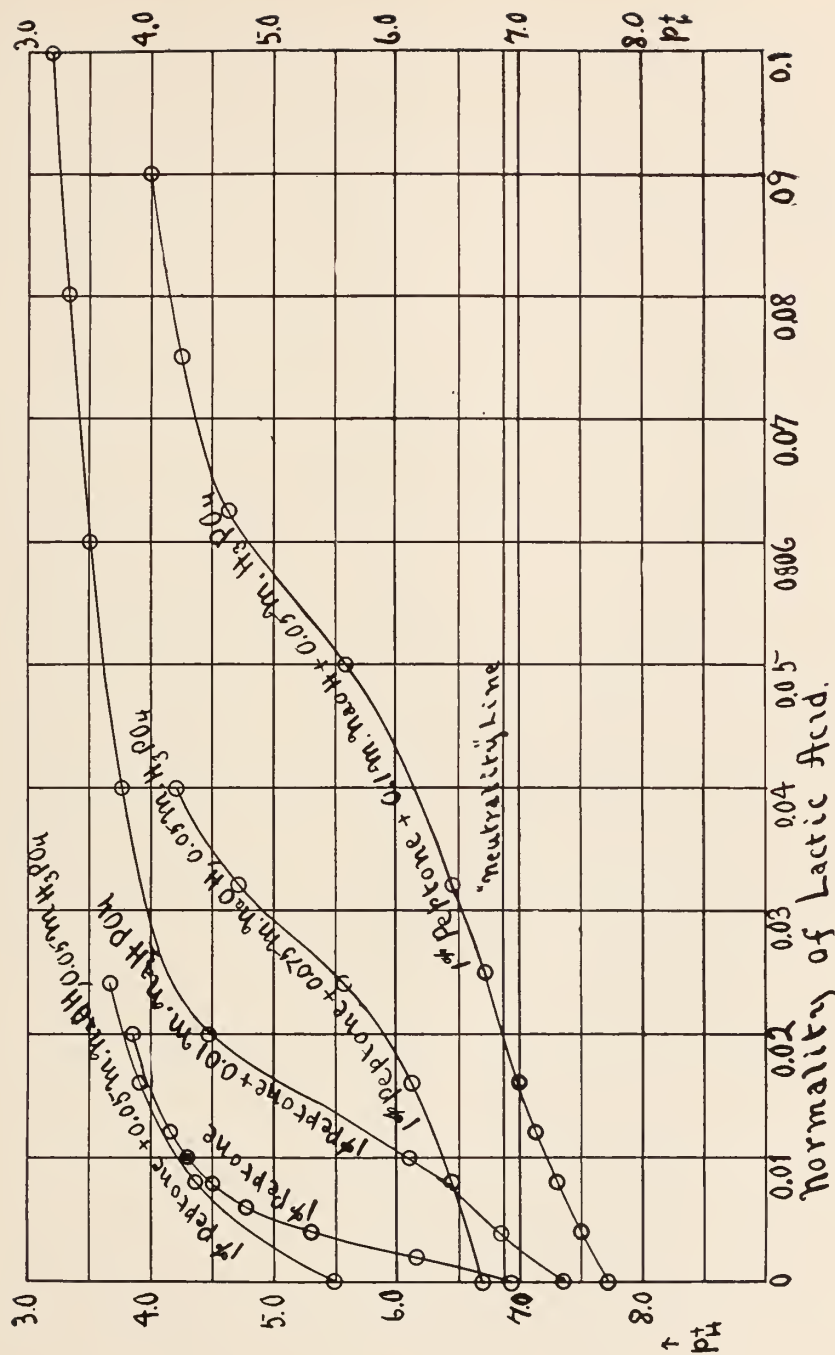


Chart 4.—Titration curves of 1 percent peptone solutions containing various mixtures of primary and secondary phosphates.

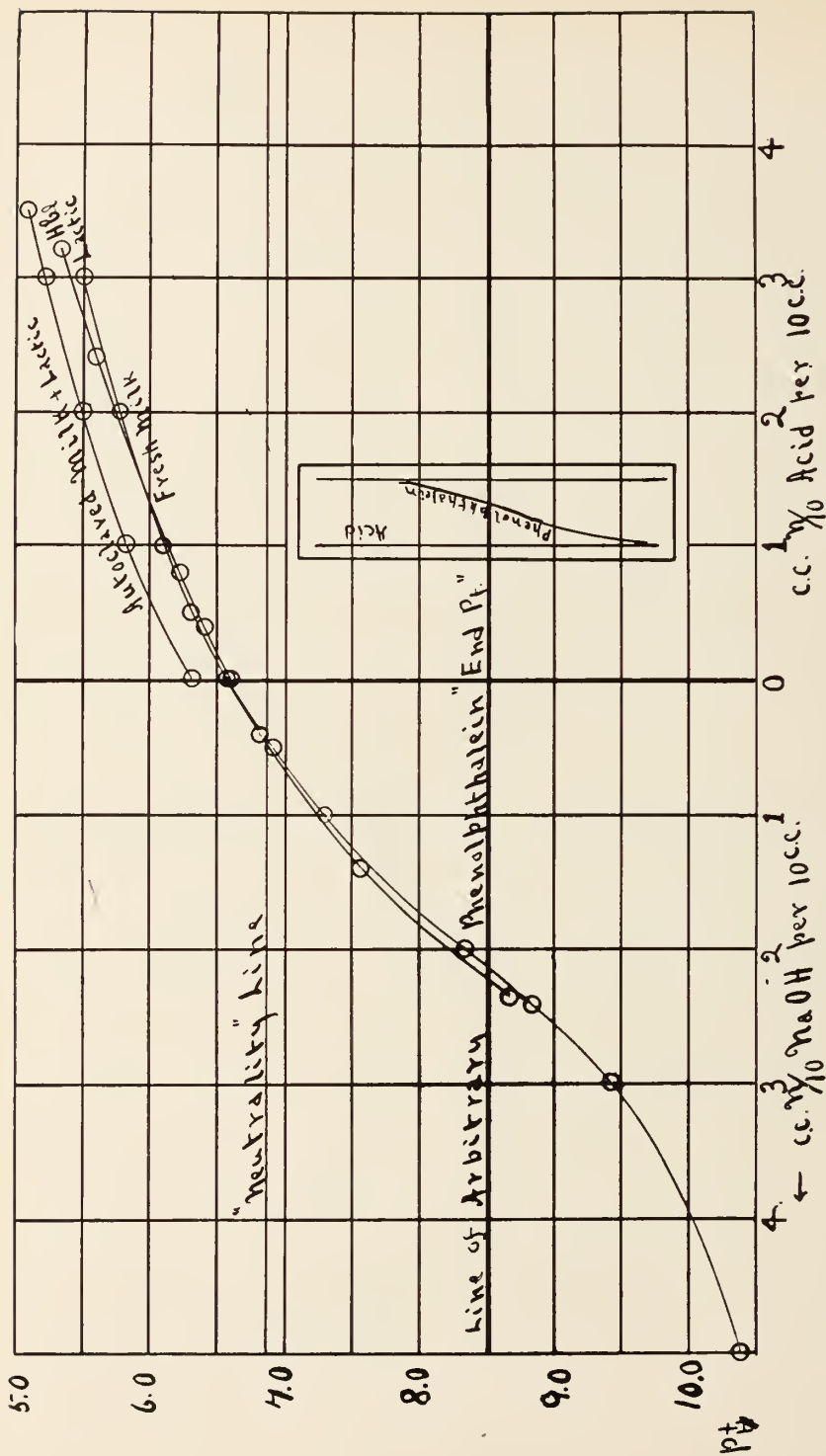


Chart 5.—Titration curves of fresh and heated cow's milk.

acid in one case and with hydrochloric acid in another. A portion of the sample which was titrated with lactic acid was titrated after heating. The heating raised the initial hydrogen ion concentration of the milk and so the titration curve of the heated sample lies above but parallel to that of the unheated portion. Titration curves of certain "modified" milks and human milk have been published in a previous paper.²⁸

TITRATION CURVES OF SOLID MEDIA

Media containing agar can not be manipulated in the hydrogen-electrode apparatus used in these studies. It is not necessary to do so however, for the buffer effect of the agar itself or of its impurities, is probably very low, and consequently agar media have approximately the same titration curves as equivalent solutions of the other constituents. That the agar itself has the properties of a "buffer" to only a slight extent seems evident from the fact that 10 c.c. of a 1.5 percent agar solution required only one drop of $n/10$ NaOH to make it distinctly alkaline to phenolphthalein, and only one drop of $n/10$ H_2SO_4 to make it distinctly acid to methyl red.

Gelatin, on the other hand, may be kept sufficiently liquid at 30 C. to be manipulated. In Chart 6 are shown the titration curves of a medium consisting of 10 percent gelatin, 1 percent Witte peptone, 0.5 percent Liebig's extract. This mixture was first titrated with NaOH before "adjustment." It will be seen that the gelatin was considerably acid, for the initial pH^+ of the media was 5.6, while the initial pH^+ of the same media without gelatin was 6.55 (Chart 3). The hydrogen ion concentration was then adjusted to $pH^+=7.0$ and the medium sterilized. The hydrogen ion concentration rose during sterilization to $pH^+=6.75$. The titrations then gave the curve marked "adjusted and sterilized." It will be noticed that at $pH^+=6.75$ this medium was +12.5 Fuller's scale. A medium of the same composition made in another laboratory and adjusted by the ordinary procedure of dilution and heating during titration, was supposed to have a "reaction" of +10 Fuller's scale. As the curve marked "From laboratory C" shows, this medium was actually only +5 Fuller's scale, or 0.5 percent. The cause of this discrepancy will be shown later.

If, now, we compare the curves of the 10 percent gelatin, 1 percent peptone, 0.5 percent Liebig's extract medium (Chart 6) with that of the same medium without the gelatin (Chart 3), it will be seen that

28. Jour. Med. Research, 1915, 31, p. 431.

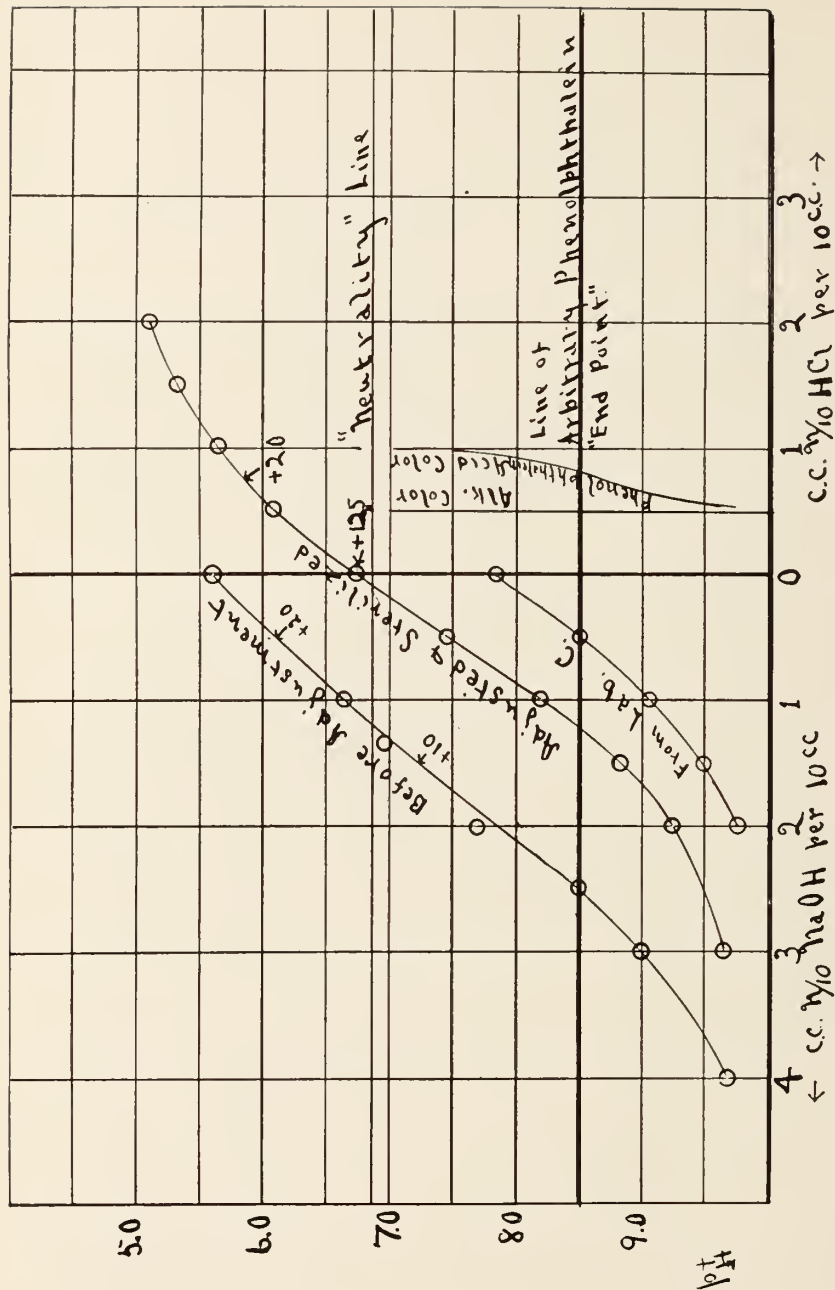


Chart 6.—Titration curves of a medium containing 10 percent gelatin, 0.5 percent Liebig's meat extract, and 1 percent Witte peptone.

the gelatin has increased the buffer effect appreciably, but that the gelatin by reason of its high molecular weight and few terminal carboxyl and amino groups is a much less effective buffer than the same weight of "peptone."

THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE COMPOSITION OF MEDIA

Much of the attention given to reactions has been concentrated upon the reaction of meat-infusion media.

It is a well-recognized fact that many proteins exist in solution only within certain narrow limits of hydrogen ion concentration. Consequently, whatever the other factors may be which influence the composition of a complex mixture like a meat infusion, the hydrogen ion concentration is important. Even in a simpler solution, such as that of Witte peptone, we may observe the precipitation of various bodies as the hydrogen ion concentration is changed by the addition of acids, alkalies, and phosphates, or other acid or alkaline salts. In the case of meat infusions the situation is more complex, for there the slightest change in the hydrogen ion concentration is often sufficient to induce a heavy precipitate. In one instance, a change from $\text{pH}^+ = 6.33$ to $\text{pH}^+ = 6.95$ was found to cause a heavy flocculation. It is therefore evident that in dealing with such a medium it will make a considerable difference whether the medium is first brought to the pink reaction of phenolphthalein and then "adjusted" from this point (Timpe's method), or whether the adjustment is made by introducing only a fraction of the alkali necessary to reach the phenolphthalein point (Fuller's method).

Frequently the precipitations induced by a change in hydrogen ion concentration are not accelerated sufficiently to become very apparent until the medium is reheated. Then there occur changes also in the hydrogen ion concentration, which we shall consider next.

THE EFFECT OF HEAT UPON THE REACTION OF MEDIA

It is well known that media generally show an increase in titratable acidity after sterilization (Hesse²⁹). It may be interesting to note first the effect of heat upon the hydrogen ion concentration of media in which very little precipitation occurs. Some data are shown in Table 2. The changes noted in the carbohydrate media are largely

due to oxidations of the sugar, which are controlled in rate both by the temperature and the hydrogen ion concentration of the solution (Henderson³⁰); also to the formation of "Melanins" (Maillard³¹).

A somewhat different phenomenon occurs upon heating a mixture like meat infusion. Here constituents are precipitated and new equilibria involving the hydrogen ions are established.³²

TABLE 2
pH⁺ VALUES OF UNSTERILIZED AND STERILIZED MEDIA DETERMINED BY ELECTROMETRIC MEASUREMENTS
AT 30 C.

Medium	Method of Sterilization	pH ⁺ Before	pH ⁺ After	Difference
1% Witte peptone.....	Autoclave	6.88	6.87	0.01
1% Witte peptone, 2% lactose	Autoclave	6.71	6.35	0.36
	Arnold	6.65	6.50	0.15
1% Witte peptone, 2% lactose, 0.050 MH ₃ PO ₄ , 0.050 NNaOH.	Autoclave	5.53	5.23	0.30
	Arnold	5.63	5.51	0.12
1% Witte peptone, 2% lactose, 0.050 MH ₃ PO ₄ , 0.075 NNaOH.	Autoclave	6.71	6.35	0.36
	Arnold	6.65	6.50	0.15
1% Witte peptone, 2% lactose, 0.050 MH ₃ PO ₄ , 0.160 NNaOH.	Autoclave	7.75	—	—
	Arnold	7.72	7.42	0.30
1% Witte peptone, 1% dextrose, 0.5% K ₂ HPO ₄	Arnold	7.43	7.23	0.20
5% Witte peptone, 2% dextrose.....	Arnold	6.75	6.63	0.12
5% Witte peptone, 2% dextrose, 0.05 MH ₃ PO ₄ , 0.050 NNaOH.	Arnold	6.55	6.46	0.09
5% Witte peptone, 1% dextrose.....	Arnold	6.83	6.68	0.15
5% Witte peptone, 1% dextrose, 0.05 MH ₃ PO ₄ , 0.050 NNaOH.	Arnold	5.87	5.57	0.30
5% Witte peptone, 1% dextrose, 0.05 MH ₃ PO ₄ , 0.075 NNaOH.	Arnold	6.55	6.40	0.15
5% Witte peptone, 1% dextrose, 0.05 MH ₃ PO ₄ , 0.100 NNaOH.	Arnold	7.19	6.94	0.25
Skimmed milk	Autoclave	6.57	6.31	0.26
Skimmed milk	Autoclave	6.58	6.14	0.44

An infusion medium containing $\frac{1}{2}$ percent K₂HPO₄ brought to pH⁺ = 7.19 before sterilization changed to 6.70 after sterilization. Another portion brought to 8.52 changed to 7.72 after sterilization. In each case the change in hydrogen ion concentration was accompanied by a heavy precipitation.

30. Jour. Biol. Chem., 1911, 10, p. 3.

31. Compt. rend. Acad. d. sc., Paris, 1912, 154, p. 66.

32. Biochem. Ztschr., 1912, 44, p. 157.

Deeleman³³ recognized that the titratable acidity changes upon sterilizing media and therefore corrected his media by adding sterile acid or alkali after the medium was sterilized.

Increase in temperature also accelerates the solution of glass. In certain instances this may become serious, especially when synthetic media, poor in "buffers," are sterilized in new vessels of "soft" glass. In the majority of cases, however, this source of error is far outweighed and does not merit the emphasis placed upon it by W. Hesse³⁴ and his son.²⁹

THE FALLACY OF TITRATING HOT MEDIA

Quite aside from any chemical change produced upon heating a medium, an elevation of temperature is accompanied by a change in the dissociation of the components of the medium. The increase in the dissociation of water is enormous. There may be an alteration also in the equilibrium of the tautomeric forms of the indicator used. The effect of all these influences is to shift the titer. In certain simple cases, it may be possible to establish a precise relation between the titer of a hot solution and that of a cold solution (Bjerrum¹¹), but for solutions as complex and variable as culture media it is impossible to do so. Redfield³⁵ has shown the great variations which may be procured by titrating peptone solutions at different temperatures. It is needless to add that, since titrations in the last analysis are based upon the attainment of a certain hydrogen ion concentration as shown by the tint of an indicator, the titration of a medium at 90-100 C. furnishes data of no exact significance at ordinary incubation temperatures. There is no procedure which deserves severer criticism than the titration of hot media. Aside from the fact that it is irrational, it has ruined the availability of an enormous body of data to be found in literature. Unless the exact procedure is given, one does not know the meaning of a +10 or a +15 reaction. But, even tho it were known, if the determinations were made by hot titrations, it would be impossible, except after laborious research, to translate these values by means of titration curves into hydrogen ion concentrations.

As an illustration, a 10 percent gelatin, 1 percent peptone, 0.5 percent meat extract medium was procured from a laboratory where the titrations were made after dilution and while the solutions were hot. This medium was reported to have a titratable acidity of +10

33. Arb. a. d. k. Gsndhtsamte, 1897, 13, p. 374.

34. Ztschr. f. Hyg., 1893, 15, p. 183.

35. A Study of Hydrogen Sulphide Production by Bacteria, Cornell Thesis, 1912.

Fuller's scale before sterilization. After sterilization, it should have been higher. Careful determinations with the hydrogen electrode and the plotting of a "titrative curve" (Chart 6) showed the acidity titratable to $\text{pH}^+ = 8.50$ to be $+5.0$. A careful titration with NaOH of the undiluted medium at room temperature and in the presence of phenolphthalein gave $+6.0$. Boiled in a vessel with a Bunsen valve, cooled, and titrated at room temperature, it gave $+6.0$. A similar titration of the undiluted medium near its boiling point gave $+10$. Five c.c. diluted to 50 c.c. with conductivity water and titrated hot gave $+19$. According to "official" methods, $+19$ should be the "true reaction." According to procedures sometimes used, $+6$ is the "true reaction." According to the temperature used and the end point assumed, the "true reaction" can be said to be anywhere between $+4$ and $+20$.

From the same laboratory, other batches of this medium were obtained, all of which were supposed to have been $+10$ before sterilization and all of which should have been higher than $+10$ after sterilization. By accurate determinations with the hydrogen electrode, they were found to be $+7$, $+5$, $+8$, $+9$, $+2$, $+9$, at 30 C. when reckoned from $\text{pH}^+ = 8.50$ as the "end point" of phenolphthalein.

THE INADEQUACY OF THE TITRIMETRIC METHOD

In the previous pages, it has been shown that: (1) Since the titration curves of culture media do not fall precipitously through the zone of hydrogen ion concentrations within which phenolphthalein changes color, there is no true "end point" in the commonly accepted meaning of that term.

(2) If a precipitous drop in the curve were found, it would indicate that in this region, at least, a very slight error in the amount of alkali added would alter the hydrogen concentration enormously. Such a medium would be practically useless for the cultivation of bacteria sensitive to changes in hydrogen ion concentration.

(3) It is necessary to assume some tint of phenolphthalein as a standard if the titrimetric method is to be accurate. The present paper color standard has no physical significance for the problem at hand, while the tint assumed by a definite amount of phenolphthalein in a standard solution of known hydrogen ion concentration has a precise physical significance.

(4) The tint of phenolphthalein at $\text{pH}^+ = 8.50$ being assumed as a standard, it has been shown that, when media are "corrected" from

this point by the titrimetric method, the degrees (Fuller scale) correspond to very different hydrogen ion concentrations in various media.

(5) Altho different batches of the same medium might be brought to the same hydrogen ion concentration by accurate titrations, this can not be done if the temperature of titration is varied. Furthermore, the titer obtained while a medium is hot has no precise meaning when the datum is applied at incubation temperature.

In short, when a medium is titrated, all that is accomplished is to reach a more or less definite hydrogen ion concentration as determined by the color of an indicator. The amount of alkali required to obtain this hydrogen ion concentration depends upon the original hydrogen ion concentration of the medium and upon the buffer content of the medium. The addition of alkali short of the amount necessary to produce a given tint of phenolphthalein leaves the medium at a hydrogen ion concentration which is indeterminate, unless the titration curve is known.

THE COLORIMETRIC METHOD

It is quite evident that if a medium is to be adjusted to $\text{pH}^+ = 8.50$, this point can be obtained accurately by simply adding alkali until the color of the medium with phenolphthalein present matches the color of a standard solution of $\text{pH}^+ = 8.50$ with phenolphthalein present. If it is desired to bring the medium to some hydrogen ion concentration outside the range of phenolphthalein, another indicator useful within the desired range may be chosen. Next to phenolphthalein, the indicator most commonly used is litmus. Unfortunately litmus, according to Walpole¹⁹ and others, can be used for this purpose only with a considerable error. But in view of the fact that a fair success has been attained in the cultivation of bacteria in media adjusted to variable hydrogen ion concentrations by the titrimetric method, it is doubtless true that the error in adjusting to some tint of litmus is of no great consequence.

A complete set of indicators suitable for the accurate adjustment of culture media, together with a study of the difficulties involved in the procedure, are being investigated in this laboratory.

GENERAL CONSIDERATIONS

It is evident that media, as they are corrected in practice, must vary considerably in their hydrogen ion concentrations. Yet a considerable degree of success in cultivating bacteria seems to have been

attained. The question then arises whether the reaction of a medium is so important as frequent assertions would lead one to believe. In attempting to answer this question, one may say at once that the initial hydrogen ion concentration, if it varies within certain limits, is not at all important so far as the mere growth of certain organisms is concerned. In this laboratory, we have had occasion to grow *Bacillus coli* and certain streptococci in media with pH^+ values varying from 5.5 to 9.0 and have obtained good growths in all. With favorable food supplies, it is probable that many other species can flourish within the same limits. In such cases, the hydrogen ion concentration of the medium makes itself felt not at all in an entire suppression of activity, but in the rates of different fermentations. We then find, as Blumenthal³⁶ did as early as 1895, that the acidity or alkalinity of the medium has an appreciable influence on the relative proportions of the end products of fermentation or putrefaction.

On the other hand, certain species, notably certain pathogens, are reported to be very sensitive to slight changes in titratable reaction. When we are considering the effect upon such sensitive organisms, we must take into consideration the medium as well as the so-called titratable acidity. A titratable acidity of 10 in one medium may furnish an optimal hydrogen ion concentration, while 10 in another medium might be unendurable to any organism. It is certainly significant that the beef infusion media, corrected by customary methods, have hydrogen ion concentrations varying between the neutral point of water and that of blood, and that the buffer effect of infusion media is so great that no great change is made in the hydrogen ion concentration by a considerable variation in the titratable acidity. From such considerations we may conclude that in certain instances the influence of reaction has been overestimated because the experiments were conducted in buffer-poor media; while in other instances the effect of reaction has been underestimated because considerable variations in added acid or alkali had little influence on the hydrogen ion concentration of richly buffered media.

In other cases in which the hydrogen ions have been considered, their influence has not been demonstrated or disproved, because their concentration has been calculated on the basis of the dissociation of acids in pure water, and no account has been taken of the "buffers." Some of the work on yeasts seems to have suffered from this oversight.

36. Ztschr. f. klin. Med., 1895, 28, p. 223.

An instance in which considerable variations in hydrogen ion concentration would probably have little effect is in making counts of the bacteria found in milk. As a rule, the initial spoilage of milk is accompanied with an acid fermentation, and the great majority of the organisms found in milk during the early stages of the spoiling are more or less acidophilic and capable of flourishing in a considerable range of hydrogen ion concentration. It is therefore almost certain that if the food supply in a meat extract broth were comparable to that in a meat infusion broth, for the organisms in question, the two media would not differ greatly in value even if the initial titratable acidity in both were the same and, consequently, the hydrogen ion concentration of one were higher than that of the other. If, however, the flora of a milk is to be investigated in detail, it is possible that the initial reaction of the media may have an appreciable influence upon the relative number of some forms which appear upon a plate; for here relative rates of growth are of chief importance. A striking instance of this is given by Rogers and Ayers.³⁷

The possibilities are that for the cultivation of the majority of bacteria it will do quite as well to leave many media unadjusted and thereby insure a more constant composition.

On the other hand, if many organisms are as sensitive as innumerable statements in the literature imply, an entirely new basis of adjusting reactions will doubtless have to be devised before comparable data can be obtained.

It certainly is suggestive to remember that the blood contains a powerful mechanism for the regulation of its hydrogen ion concentration (Henderson³⁸). It has been shown that small alterations in this concentration are attended by serious consequences to the cells and to the enzymes which find blood their natural medium. It was a similar consideration which led Henderson and Webster³⁹ to propose their phosphate mixture for the regulation of the hydrogen ion concentration of bacterial cultures.

Likewise, Sørensen and Palitzsch⁴⁰ have shown the remarkable constancy of the hydrogen ion concentration of the oceans, and Henderson⁴¹ has vividly described the biologic importance of the carbonate equilibrium which makes this constancy possible.

37. U. S. Dept. Agriculture, 1910, Circular 153.

38. *Ergebn. d. Physiol.*, 1909, 8, p. 254.

39. *Jour. Med. Research*, 1907, 16, p. 1.

40. *Biochem. Ztschr.*, 1910, 24, p. 387.

41. *The Fitness of the Environment. An Inquiry into the Biological Significance of the Properties of Matter*, 1913.

Bacteria in the great majority of their natural habitats find one or more buffers which tend to neutralize the acid or basic products of their metabolism and thus permit an extension of bacterial action. When, therefore, bacteria are transplanted to artificial media, they should be provided there, not only with proper food, but with buffers to protect them from the toxic effect of the acid or basic products of their metabolism. Not only must this be done, but, if the maximal rate of activity is to be attained, the organisms must find the hydrogen ion concentration of the medium suitable for the optimal activity of their enzymes and consistent with the stability of their colloidal structures. In many instances a considerable variation in hydrogen ion concentration may be tolerated, but only a narrow range may be suitable for optimal rates of these processes, upon which many commercial and academic questions depend.

CHART REPRESENTATION OF PHYSIOLOGICALLY IMPORTANT HYDROGEN
ION CONCENTRATIONS

The data already presented will have a clearer significance if we have before us a graphic representation of the hydrogen ion concentrations of certain biologic fluids and the zones of enzyme action. Such a representation has been attempted in Chart 7. This has been compiled for the most part from the data given in Michaelis' book. In representing the hydrogen ion concentration (as pH^+ values) of blood, for instance, it must not be understood that blood is always at the point indicated. It varies slightly, cows' milk slightly, muscle juice in proportion to the degree of autolysis, and feces with the diet. The extent of the variations will be found in original papers. Likewise, the representations of the zones of enzyme action are approximations, designed only to give a general idea.

This chart is similar to, but much less complete than Walpole's.²⁰ In one respect, however, it is believed to have an advantage. Walpole gives only the optimal points for various enzymes and leaves the reader rather confused as to the zone of hydrogen ion concentration throughout which any particular enzyme can operate. In the present chart, the optimum for trypsin, for instance, is shown at $\text{pH}^+ = 8.00$, but the enzyme still preserves about 50 percent of its activity at $\text{pH}^+ = 6.6$, and reaches minimal activity only at about $\text{pH}^+ = 5.0$.

It is important to note the breadth of these zones, for if bacterial enzymes are similar to the better known enzymes, they are operative

over a considerable range of hydrogen ion concentration. At the same time, it is very important to recognize that altho an enzyme may be active outside its optimal range, its rate of action may be enormously diminished. In symbiotic processes, especially, rates are all important.

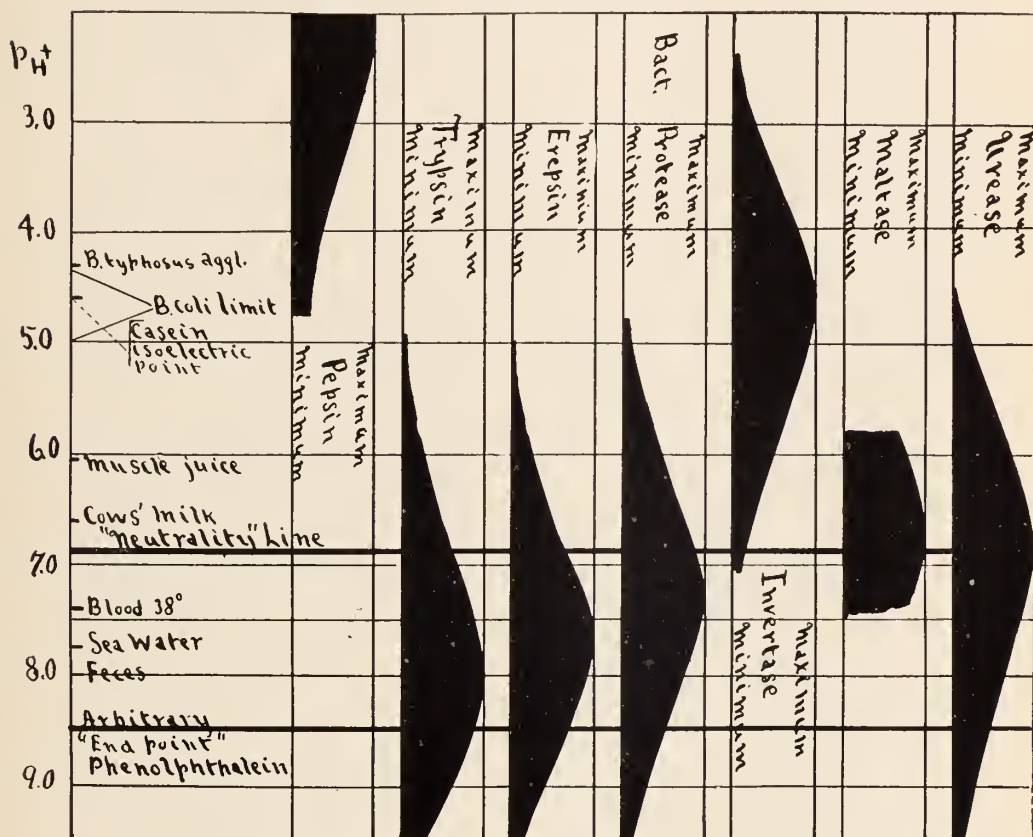


Chart 7.—Representation of hydrogen ion concentrations of physiologic importance, and of the approximate range and degree of activity of several enzymes.

SUMMARY

The principles of titration have been briefly reviewed. The hydrogen ion concentrations of various culture media before and after the addition of successive quantities of alkali and acid have been determined by means of the hydrogen electrode. From the data so obtained, "titration curves" have been constructed which show (1) the amount

of acid or alkali which it is necessary to add to certain media in order to reach a given hydrogen ion concentration such as would be indicated, for instance, by a certain tint of phenolphthalein; (2) the buffer effect of various media; (3) the hydrogen ion concentrations of different media adjusted to various degrees of reaction (Fuller's scale). It is shown that adjustment of different media to the same degree of reaction (Fuller's scale) may result in media having widely different hydrogen ion concentration.

The titrimetric method is further shown to be vitiated by the custom of titrating media while hot.

The fallacies of the titrimetric method of adjusting culture media have been shown to point to the advisability of its abandonment and to the substitution of a colorimetric method.

The influence of hydrogen ion concentrations upon the composition of complex media has been touched upon. Some data have been presented showing the influence upon the hydrogen ion concentration of oxidations of carbohydrates and precipitation of proteins when accelerated by high temperatures.

The significance of the proper adjustment of the hydrogen ion concentration of media has been discussed and certain points illustrated by means of a chart representation of hydrogen ion concentrations of physiologic importance.

THE CHARACTERISTICS OF BACTERIA OF THE COLON TYPE OCCURRING ON GRAINS *

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INTRODUCTION

It has been recognized for many years that bacteria of the colon-aerogenes type have a wide distribution outside of the digestive tract.

In 1899 Laurent¹ expressed the opinion that *Bacillus coli communis* may become parasitic on the tubers of the potato, and at about the same time Klein and Houston² noted the occurrence of both typical and atypical colon bacteria on grains coming from different parts of the world. Papatotiren³ found *Bacillus coli* on grains of various kinds, altho it was not invariably present. Dügge⁴ found bacteria on the surface of plants, fruits, and seeds in numbers too great to be accounted for by contamination from dust of the air. *Bacillus coli* was among the varieties occurring less frequently. In this country, Metcalf⁵ reported the occurrence of *Bacillus coli* on the surface of fruit, flowers, and various grains. In this work the possibility of fecal contamination was admitted; but Prescott,⁶ who made an extensive study of the distribution of the organism in question on grains, found it not only on grains growing in fertilized fields, but also on grains growing under conditions which made the possibility of direct contamination with fecal matter remote. The work of Winslow and Walker⁷ did not confirm the results obtained by Prescott and other investigators. No typical *Bacillus coli* cultures were obtained from 178 samples of grain or 40 samples of grass.

The general assumption has been that the colon-aerogenes group was widely distributed throughout nature, but that its normal habitat was the digestive tract, and its occurrence elsewhere due to a more or less direct contamination with fecal matter. It is held by many bacteriologists that multiplication is confined almost exclusively to the digestive tract, and that the occurrence on grains is due to contamination by dust particles. This view is not in accord with the work of Johnston,⁸ who found a bacillus, which he could not differentiate from *Bacillus coli*, causing a bud rot of certain palms. Burri and Andrejew⁹ believed that a strain which they found growing on grass was distinct and could be

* Received for publication May 3, 1915.

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1. Inst. Past. Ann., 1899, 13, p. 1.

2. Twenty-Ninth Ann. Rep. Gt. Brit. Govt. Bd. Suppl. Rep. Med. Off., 1899-1900, p. 593.

3. Arch. f. Hyg., 1901-1902, 41, p. 204.

4. Centralbl. f. Bakteriöl., II, 1904, 12, pp. 602, 695; 13, pp. 56, 198.

5. Science, 1905, 22, p. 439.

6. Ibid., 1902, 15, p. 376; Biological Studies by the Pupils of William Thompson Sedgwick, Boston, 1906.

7. Science, 1907, 26, p. 797.

8. U. S. Dept. of Agr., Bureau of Plant Industry, 1912, Bull. 228.

9. Centralbl. f. Bakteriöl., I, O., 1910, 56, p. 217.

distinguished from the fecal type by physiologic reactions. Prescott, while he considers the grain and fecal types identical, believes that there is actual growth on the grains. The failure to differentiate the colon-like organisms found on grain from those occurring in the intestinal tract is not surprising in view of the chaotic condition existing in the classification of this group. The varieties which have been recognized have been based on characters which are unimportant, or admittedly arbitrary.

In addition to its very apparent scientific interest, the problem of the identity or plurality of the strains of the colon-aerogenes group occurring in the intestines and on grains has a sanitary importance in relation to the significance of the occurrence of this organism in water and milk. If the surface of grains, fruits, and plants in general is the habitat of a distinct variety of this group, the mere demonstration of colon organisms in milk or water loses much of its significance, and becomes more than ever only presumptive evidence of contamination with fecal matter.

In our earlier work,¹⁰ we have shown that while the colon bacteria occurring in bovine feces belong to one sharply circumscribed variety, there occur in milk two or more distinct physiologic families. It is true that this conclusion in regard to the bacteria of bovine feces is based on the examination of sixteen samples coming from a single herd, but the results obtained from the 150 cultures studied were so consistent that it is believed they are characteristic. In this paper, we propose to show the characteristics, particularly the physiologic functions, of a collection of colon-like bacteria obtained from representative samples of grain, and by means of these characteristics to group them into types which may be compared with the type found in the bovine digestive tract.

SOURCE OF CULTURES

Of the 166 cultures included in this study, 75 were obtained from corn, 6 from barley, 30 from wheat, and 41 from oats. These were all dried grains, obtained from the grain inspection laboratory of the Bureau of Plant Industry through the kindness of Dr. Duvel and his assistants. The samples were taken in most cases from cars of grain on the track and were transmitted to our laboratory without breaking the packages. Cultures were thus obtained from thirty-three samples of dried grains coming from five states.

In addition to the cultures from the dried grains, fourteen cultures were secured from two samples of green oats, collected in the vicinity

10. Jour. Infect. Dis., 1914, 14, p. 411; 15, p. 100.

of Washington. The heads of the grain were taken without handling and the husks opened with aseptic precautions, so that the growth came from the immature, milky grain. In the case of a third sample, transferred in a similar way to dextrose broth, no growth was obtained.

It is obvious that there was abundant opportunity for contamination of the samples of dried grains, both from dust of the air and from the hands of the various people who handled the grain. There can be no assurance that the colon-like organisms found on these samples actually grew on the grain. In the case of the two samples of green oats, it is very probable that the bacteria were actually growing in the milk of the grain. The conclusive evidence which will be presented in this paper of the occurrence on grains of a distinct type demonstrates that these bacteria are not air-borne contamination from fecal sources.

The cultures were obtained by placing a few grains of the sample in dextrose broth, incubating at 30 C. over night, and plating the broth on infusion agar. Representative colonies were fished from the plates to dextrose broth with inverted tubes, and four or five of the tubes showing gas were replated.

Altho the fermentation of dextrose rather than lactose was used as a basis of isolation, nearly all of these cultures would respond to the usual tests for the colon group. With the exception of a small sub-group, all gave the usual presumptive tests for colon, including the formation of gas in lactose bile.

METHODS OF STUDY

Gelatin Liquefaction.—The liquefaction of gelatin was determined by spreading a drop of a broth culture on the surface of infusion gelatin in a tube, marking the level of the surface of the gelatin on strips of paper on opposite sides of the tube, and incubating for twenty days at 20 C. If liquefaction occurred, the amount was measured and expressed in millimeters of liquefaction.

Indol.—The production of indol was determined in a medium consisting of 1,000 c.c. water, 0.3 gm. of tryptophan, and 5 gm. of K_2HPO_4 . Cultures were grown in this medium two days at 30 C. The test for the presence of indol was made as follows: To the medium was added, drop by drop to avoid mixing it with the medium, 1 c.c. of a 2 percent alcoholic solution of p-dimethyl-amidobenzaldehyd, then a few drops of concentrated hydrochloric acid. The presence of indol was indicated by a violet color.

Capsule.—In the earlier investigations no attempt was made to determine the presence of a capsule, but on account of the importance ascribed to this characteristic by some bacteriologists, it was decided to include this in the routine examination of these cultures. With the methods at first employed, it was very difficult to get consistent results. Later we adopted the Welch method. This, as outlined to us by Dr. Harris, of the University of Chicago, is as follows: Make a smear from an eighteen-hour milk culture. Dry. Fix

over the flame. Wash with glacial acetic acid. After five seconds, wash several times with carbolfuchsin. Wash well with 0.9 percent solution sodium chlorid and mount in this solution for examination. While this method was more satisfactory, it was possible to obtain consistent results only by careful manipulation in the preparation of the slides.

In certain groups, the capsule appeared large and unmistakable. This was especially true of Type E. In Type D, on the contrary, in which the capsule is recorded positive for 30 percent of the cultures, it was small and often faint and indefinite. Probably this group is constant in the possession of a small capsule which often fails to be demonstrated by the method employed.

Pigment Formation.—The members of the colon group are not usually considered as pigment formers, altho pigmented, colon-like cultures have been reported.¹¹ One of the cultures isolated from bovine feces was pigmented. Such a large proportion of the grain series now under consideration showed some color that it was decided to determine the pigment with more care than is usually attempted. In most of the cultures, the amount of pigment was so small, especially in the young culture, that it would not be noted, or at least would be classed as a "dirty white." In a few cultures, however, the color was distinct, even in the young culture.

The pigment was compared with a standard by growing an agar culture fifteen days at 20 C. and spreading as much as possible of the growth on white drawing paper. Better results were obtained by comparing the standard with the moist culture than with one which had dried on the paper, because the amount of pigment was so small that when dried it did not cover the white of the paper, and it was difficult to get consistent results.

The standards used were the plates in Ridgway's Color Standards and Nomenclature, which have been found valuable for this purpose. A word of explanation of the symbols used to designate these standards may make Table 7 more intelligible. Colors are designated by letters, as "oy," which indicates a color made up of 65 percent orange and 35 percent yellow; "yoy," 47 percent orange and 53 percent yellow; or "oyy," 25 percent orange and 75 percent yellow. Variations from the pure colors are obtained by mixtures of neutral gray. For instance, 17oy is a pure color; 17'oy contains 32 percent neutral gray; 17''oy, 58 percent neutral gray. Finally, tints, designated by lower-case letters, are obtained by mixing varying proportions of white with the pure colors, and shades are obtained by mixing black. The tint nearest the pure color is designated by the letter b, that nearest pure white by f; the shade nearest the pure color is designated by the letter i.

Reference to the plates will show that the difference between the corresponding lighter tints of the various orange-yellow colors is so small that the identification of the pigment of a culture with one or the other does not indicate a marked difference. In making up Table 7, only the colors, tints, and shades were included with which cultures were identified.

Fermentation.—The value of the fermentation reactions for the division of the colon-aerogenes group into its component sub-groups is so well established that it need not be discussed in this paper. There is much uncertainty and difference of opinion, however, as to the test substances to be used in fermentation tests. In our opinion, no definite schedule of test substances can be predetermined, as the particular ones giving the most characteristic reactions will vary with the group of cultures under observation. These should

include representatives of the different types of compounds which may serve as sources of carbon for zymogenic bacteria. Therefore we included simpler sugars, such as dextrose; more complex sugars, such as saccharose, lactose, and raffinose; polysaccharids, as starch and inulin; alcohols, such as mannite, glycerin, adonite, and dulcite; and one glucosid, salicin.

The fermentation of a substance may be determined by the evolution of gas or by the production of acid. In our work, we have confined the study of fermentation by gas production to dextrose, and have depended on the acidity determination for the fermentation of other test substances.

Under certain circumstances which have not yet been definitely determined, the acid from the fermentation of sugar may be masked by a secondary alkaline production, sufficient in some cases entirely to obscure the acid formation. In one small group of this collection, the lactose broth tubes at the end of seven days were only slightly more acid than the blank, altho all of the cultures gave gas in lactose bile. In no case was the titration of the culture less than that of the blank, altho this was usually the case with broths in which there was no fermentation. Where positive evidence of the fermentation of a sugar was obtained in another way, the negative evidence of the titration was disregarded and in the correlation tables the culture was included with the positive reactions. If, for instance, the titration of lactose broth was negative, while the lactose bile fermentation tubes showed gas, the cultures were considered to be lactose positive.

Kligler¹² considers the reaction with salicin very significant. We included this glucosid with the test substances used in some of our earlier work, but did not use it on the fecal series because we found that it was fermented by such a large percentage of the cultures that it seemed to have little value for diagnostic purposes. Altogether, 279 cultures were tested for their ability to ferment salicin. Of these 264, or 94.6 percent, fermented salicin while only fifteen failed. Notwithstanding this evidence, salicin was included in this series in order that the results might be more comparable with those obtained by Kligler. It was found however that only one or two of the entire collection failed to ferment salicin. In compiling the tables, the results obtained with salicin and dextrose were omitted for the sake of brevity.

In studying the gas production of these bacteria, we again used the mercury vacuum pumps and the technic described in our former papers.¹³ The only modification of the procedure described in our last paper was the use of a Sprengel pump to complete the initial evacuation of the bulbs begun with a "Geryck" oil pump. The gas was analyzed over mercury in carefully calibrated burettes. All gas volumes were reduced to normal temperature and pressure dry.

The medium was of the same composition as that employed in our last series of determinations with the colon cultures from cow feces. It was made as follows: Ten grams dextrose, 10 gm. Witte's peptone, and 5 gm. Kahlbaum's dibasic potassium phosphate (K_2HPO_4) were added to 800 c.c. distilled water. This mixture was heated with occasional stirring for twenty minutes over steam. It was then filtered through a Schleicher and Schüll No. 588 folded filter. The filtrate was cooled to 20 C. and then made up to 1,000 c.c. The dextrose with which this medium was made up, was, in this series, a sample of exceptionally high purity given us by Dr. Hudson of the Bureau of Chemistry.

12. Jour. Infect. Dis., 1914, 15, p. 187.

13. Jour. Infect. Dis., 1914, 14, p. 411; 15, p. 100.

The incubation temperature was 30 C., maintained constant $\pm 0.1^\circ$, except on two occasions both of which fortunately occurred at the end of an incubation period. We did not strictly adhere to the seven-day incubation period which we used in former series because, in every case we have studied, gas production by similar organisms ceased within one hundred hours after inoculation. A variation of one or two days from the seven day incubation period is therefore not likely to have affected our results.

All analyses made, except four, are included in the tables. In two of the four omitted, gross errors were definitely detected in the determination of carbon dioxid. The total gas in these cases was for *qy*, 28.8 c.c., and for *op*, 27.8 c.c. In the other two analyses, gross errors in the determination of hydrogen were definitely detected. The carbon dioxid contents of these cases were for *qq*, 69.8 percent and for *qe*, 71.4 percent. For the duplicate determinations with these organisms, see Table 3.

The separation of the analyses into three tables was made upon the basis of very distinct differences in the ratio $\text{CO}_2 : \text{H}_2$. In Table 1 are grouped the eight cultures the ratios of which were low, agreeing with those of that large group previously isolated by us from bovine feces.

In Table 2 will be found the analyses of gas from seven cultures which produced carbon dioxid but no hydrogen. The ratio $\text{CO}_2 : \text{H}_2$ in these cases became infinity (∞ —a symbol which we shall retain in spite of its pedantry for its convenience in tabulation and description.)

All of the remaining cultures gave a ratio above 1.90, their general characteristics being tabulated in Table 3. The cultures are subdivided in this table into the four types which will be described later. Under each type are placed, first, those cultures which showed very little or no variation from the type, and next, the cultures which differed from the type in one or more significant characteristics. We shall therefore refer to the cultures and analyses which fall in Tables, 1, 2, or 3 as members of the "low ratio group," " ∞ group," or the "high ratio group," respectively.

The averages of the values listed in the three tables of analyses are given in Table 4, which is self-explanatory in all points except the "nitrogen" values. These were calculated by subtracting the sum of carbon dioxid and hydrogen from the total volume and assuming that the residual gas was nitrogen.

In order to visualize the distinct differences in the gas production of these three groups, the individual analyses have been plotted in Chart 1. In this chart, the abscissa of each dot represents the cubic centimeters of CO_2 produced, and the ordinate the cubic centimeters of H_2 . The diagonal passing through the origin is the $1.06 x = y$ line along which

TABLE 1
CHARACTERISTICS OF CULTURES WITH RATIO $\text{CO}_2/\text{H}_2 = 1.66$ (TYPE A)

Cul- ture	Cap- sule	Lique- faction of Gelatin	Indol from Tryptophan	Pigment	Fermentation Reactions Expressed as c.c. Normal Acid per 100 c.c. Medium								Volume of Gas in c.c.	Per- centage CO ₂	Per- centage H ₂	Ratio CO ₂ H ₂		
					Saccha- rose	Lac- tose	Raffi- nose	Starch	Inu- lin	Man- nitol	Glyc- erin	Ado- nite					Dul- cite	
oq	—	0	+		19yoya	-1.30	6.10	3.65	-0.60	0.00	4.15	1.70	4.80	4.30	11.80	51.0	48.1	1.06
or	+	0	+		19yoyb	6.60	5.00	4.70	-0.70	0.00	4.20	1.05	0.00	-1.20	8.03	51.5	48.2	1.07
ou	—	0	—		19yoya	-0.70	4.20	0.90	0.00	0.00	3.45	0.95	0.00	0.00	8.04	50.7	47.9	1.06
st	—	0	—		19yoya	0.70	0.70	2.30	0.00	-1.10	2.80	0.75	0.00	-1.55	12.58	51.4	48.5	1.06
sv	—	0	—		19yoya	0.90	0.00	3.00	0.00	0.00	3.35	0.60	-0.95	-1.10	11.94	51.8	48.4	1.07
te	—	0	+		19yoya	-0.25	2.35	1.35	-0.30	-0.85	3.80	0.95	4.25	3.70	15.06	50.8	48.8	1.04
tf	—	0	—		19yoya	3.85	1.95	2.55	0.00	3.85	3.10	0.75	0.00	1.55	16.75	51.5	48.2	1.06
ue	—	0	+		19yoyl	-0.85	4.70	0.35	-0.30	-0.95	4.00	0.55	3.85	3.40	14.67	51.0	48.6	1.05
															16.02	51.0	48.6	1.05
															8.51	52.0	47.5	1.10
															12.00	51.4	48.0	1.07
															15.41	51.9	47.9	1.08
															15.82	50.7	49.2	1.03
															10.44	50.9	48.6	1.05
															11.81	50.8	48.3	1.05

TABLE 2
CHARACTERISTICS OF CULTURES PRODUCING CO₂ ONLY

[illegible]

TABLE 3

CHARACTERISTICS OF CULTURES WITH CO_2/H_2 ABOVE 1.90

Culture	Cap- sule	Lique- faction of Gelatin in mm.	Indol from Trypto- phan	Pigment	Fermentation Reactions Expressed as c.c. Normal Acid per 100 c.c. Medium							Volume of Gas in c.c.	Per- centage CO_2	Per- centage H_2	Ratio CO_2 — H_2
					Saccha- rose	Lac- tose	Raffi- nose	Starch	Inu- lin	Man- nitol	Glyce- rin	Ado- nite			
Type C															
op	—	10	—	19'yoxyb	2.80	4.10	3.55	-0.50	0.00	3.10	1.90	-1.10	27.95	70.4	29.3
ol	—	7	—	19'yoxyb	2.95	3.90	2.55	0.00	-0.90	3.40	1.35	0.00	27.18	74.2	25.5
ou	—	7	—	19'yoxyb	2.50	3.35	2.55	0.00	-0.85	1.55	1.10	0.00	31.34	71.4	27.3
pu	—	Slight	—	19'yoxyb	4.45	4.60	3.10	0.00	0.00	3.00	1.20	0.00	28.97	73.1	26.2
pr	—	Slight	—	19'yoxyb	4.35	4.60	3.60	0.00	0.00	3.30	1.00	0.00	28.18	73.1	26.5
rd	—	Slight	—	19'yoxyb	3.35	4.25	3.70	0.00	0.00	3.00	1.20	0.00	27.75	72.5	27.4
rg	—	7	—	19'yoxyb	3.75	4.05	3.70	0.00	0.00	2.60	0.80	0.00	28.22	71.6	28.3
rh	—	9	—	19'yoxyb	3.00	3.35	3.10	0.00	0.00	3.00	0.80	-0.60	27.86	72.0	27.7
ri	—	5	—	19'yoxyb	3.60	4.20	2.80	0.00	-0.70	3.25	2.00	0.00	28.02	70.8	29.1
rj	—	Slight	—	19'yoxyb	4.00	3.25	1.60	0.00	0.00	1.90	0.80	0.00	29.10	71.5	28.4
rk	—	8	—	19'yoxyb	4.10	3.65	3.65	-0.70	0.00	2.30	0.70	0.00	29.37	69.8	30.1
rl	—	Slight	—	19'yoxyb	3.75	3.20	2.65	-0.15	0.00	2.30	1.00	0.00	29.13	71.4	28.3
rm	—	8	—	19'yoxyb	4.35	3.85	2.70	0.00	0.00	1.45	1.25	0.00	27.02	72.3	27.5
ro	—	6	—	19'yoxyb	4.00	3.60	3.15	0.00	0.00	3.80	1.00	-0.95	27.33	71.3	28.7
rw	—	9	—	19'yoxyb	3.60	3.70	3.20	0.00	-0.90	3.40	1.15	0.00	27.23	73.1	26.8
ry	—	9	—	19'yoxyb	3.40	3.40	4.20	0.00	0.00	1.55	0.80	-0.70	27.48	72.0	27.7
rz	—	5	—	19'yoxyb	4.75	2.60	2.00	-0.10	0.00	3.65	1.30	0.00	28.73	73.0	26.9
sa	—	Slight	—	19'yoxyb	4.05	3.00	5.20	-0.30	0.00	0.80	1.10	0.00	27.08	73.5	26.5
sb	—	4	—	19'yoxyb	3.70	3.45	3.15	0.00	-1.10	2.30	1.40	0.00	1.75	72.9	26.8
sc	—	Slight	—	19'yoxyb	3.35	2.90	2.30	-0.35	0.00	2.80	1.50	-1.00	27.49	72.0	27.8
sd	—	6	—	19'yoxyb	4.15	3.85	3.30	0.00	0.00	3.15	1.15	-0.80	28.43	72.6	27.2
sl	—	Slight	—	19'yoxyb	4.55	4.40	3.70	-0.35	0.00	3.05	1.45	-1.00	28.00	73.8	26.1
sm	—	5	—	19'yoxyb	3.90	4.45	3.15	0.40	0.00	2.70	1.20	0.00	28.22	72.2	27.6
sz	—	8	—	19'yoxyb	4.45	4.40	3.40	0.00	0.00	2.60	1.55	-1.05	28.89	72.9	26.9
tp	—	5	—	19'yoxyb	4.40	2.75	3.60	-0.45	0.00	2.15	1.30	0.00	27.81	73.0	26.6
tr	—	9	—	19'yoxyb	4.40	2.35	3.95	-0.25	0.00	2.75	1.30	0.00	27.58	72.7	26.9
tt	—	9	—	19'yoxyb	4.35	2.45	3.65	0.00	0.00	2.60	1.30	-0.70	27.53	71.8	27.8
tl	—	Slight	—	19'yoxyb	4.55	2.95	2.90	0.00	0.00	3.40	0.80	-0.85	27.83	73.4	26.3
tu	—	5	—	19'yoxyb	4.63	2.95	3.50	0.00	0.00	2.55	1.25	0.00	27.87	73.5	26.1
ty	—	Slight	—	19'yoxyb	3.80	4.60	2.40	-0.25	0.00	3.60	1.00	0.00	28.64	69.6	29.7
tz	—	Slight	—	19'yoxyb	4.05	4.55	3.25	0.00	0.00	3.40	1.35	0.00	28.50	71.2	28.5
ua	—	Slight	—	19'yoxyb	4.00	4.40	3.25	0.00	0.00	3.15	1.50	0.00	26.28	74.1	25.7
ub	—	5	—	19'yoxyb	4.15	4.30	3.30	0.00	0.00	2.55	1.35	0.00	27.81	72.8	27.0
uc	—	Slight	—	19'yoxyb	4.25	3.80	3.65	-0.30	0.00	1.80	1.30	0.00	28.30	72.0	27.8
ud	—	Slight	—	19'yoxyb	3.35	3.35	2.95	0.00	0.00	2.35	1.20	0.00	27.65	72.7	27.6
ue	—	Slight	—	19'yoxyb	3.30	3.70	3.10	-0.30	0.00	3.15	0.95	0.00	28.11	72.0	27.7
uf	—	Slight	—	19'yoxyb	3.85	3.35	3.10	-0.30	0.00	2.55	1.05	-0.75	27.98	71.5	28.3
uj	—	Slight	—	19'yoxyb	3.10	3.80	3.70	0.00	0.00	1.35	1.20	0.00	27.45	72.9	27.0
uk	—	Slight	—	19'yoxyb	3.40	3.00	3.55	0.00	-0.85	3.30	0.90	-1.00	26.40	73.1	26.6
ul	—	Slight	—	19'yoxyb	3.40	3.00	3.55	0.00	-0.85	3.30	0.90	-1.00	26.48	71.9	27.7
uh	—	Slight	—	19'yoxyb	4.20	3.75	2.80	0.00	-1.05	2.30	1.20	-0.90	26.58	73.0	26.9
Type D															
oo	—	0	—	19'yoxyd	1.55	4.65	3.40	0.00	0.00	-0.70	0.85	-1.00	30.03	70.5	29.4
ow	—	0	—	19'yoxyd	1.80	5.35	2.90	0.00	-1.00	-0.10	1.15	-0.85	31.58	71.1	28.9
ox	—	0	—	19'yoxyd	1.40	5.45	3.45	0.00	0.00	-0.40	1.10	-1.05	30.35	70.5	29.3
oy	—	0	—	19'yoxyd	1.60	5.45	1.30	0.00	0.00	-0.05	1.45	-0.80	30.08	70.1	29.7
oz	—	0	—	19'yoxyd	2.20	5.25	3.55	0.00	0.00	0.00	1.10	-1.50	30.82	70.7	29.1
oz	—	...	—	19'yoxyd	31.12	70.2	29.6
pa	—	0	—	19'yoxyd	1.85	6.95	3.50	0.00	0.00	0.80	0.85	0.00	31.79	68.7	31.1
pb	+	0	—	19'yoxyd	3.85	5.70	3.65	0.00	0.00	0.30	0.60	-0.85	30.98	69.1	31.0
pc	—	0	—	19'yoxyd	1.80	5.20	1.75	-0.15	0.00	0.05	0.60	-1.10	32.07	68.3	31.7
pl	+	0	—	19'yoxyd	1.10	3.80	2.60	0.00	0.00	0.95	1.00	-1.00	30.43	70.3	29.5

TABLE 3.—Continued

Cul- ture	Cap- sule	Lique- faction of Gelatin in mm.	Indol from Trypto- phan	Pigment	Acid Fermentation Reactions Expressed in Percentages								Volume of Gas in c.c.	Per- centage CO ₂	Per- centage H ₂	Ratio CO ₂ H ₂	
					Saccha- rose	Lae- tose	Raffi- nose	Starch	Inu- lin	Man- nite	Glyce- rin	Ado- nite					Dul- cite
os	—	0	—	21"oxyf	1.95	2.25	2.35	-0.25	0.00	1.50	1.20	4.70	-1.45	31.08	68.7	31.2	2.20
ot	—	0	—	19"oxyf	2.50	3.35	3.10	-0.35	0.00	1.05	0.80	4.65	-1.50	31.55	68.1	31.9	2.14
qv	—	0	—	21"oxyf	1.30	5.00	2.00	0.00	0.00	0.75	1.05	4.55	-1.50	32.02	68.3	31.3	2.18
qw	—	0	—	19"oxyf	1.90	5.00	1.45	0.00	0.00	0.15	0.95	4.40	-1.70	30.33	69.4	30.5	2.27
qx	+	...	—	30.27	68.9	31.0	2.23
qx	+	0	—	19"oxyf	1.65	5.05	3.35	0.00	0.00	-0.50	0.90	5.45	-1.60	31.25	68.5	31.4	2.19
om	+	0	—	None	2.75	4.15	1.95	5.85	-0.70	0.85	0.90	-0.60	-1.10	28.81	69.5	30.4	2.28
ov	+	0	—	None	3.25	4.20	1.55	5.20	-0.70	0.60	2.65	-0.70	-1.10	29.20	70.2	29.2	2.40
pv	+	0	—	19"oxyf	2.20	4.50	2.10	5.20	-0.75	1.20	1.20	-0.60	-1.10	28.29	71.8	28.1	2.55
py	+	0	—	None	2.40	4.90	3.50	5.50	-0.75	1.70	3.20	-0.80	-0.70	28.77	70.1	29.7	2.36
rq	+	0	—	None	2.40	5.05	3.55	6.20	-0.75	0.35	3.45	-0.70	-1.05	29.69	70.1	29.7	2.36
sx	+	0	—	17"oxyd	3.45	5.10	2.45	4.35	-0.45	1.55	2.25	-0.70	-0.60	30.23	68.6	31.2	2.20
pd	+	0	—	None	4.15	4.35	4.00	5.65	-0.30	0.80	0.95	-0.60	0.95	30.01	69.6	30.3	2.30
rb	+	0	—	None	2.65	4.10	2.50	5.55	-0.70	0.35	2.35	-0.55	0.70	28.47	67.2	32.8	2.04
re	+	0	—	None	2.90	4.30	3.40	2.60	-0.75	0.45	3.30	-0.60	0.85	29.14	68.0	31.8	2.14
tx	+	0	—	21"oxyf	2.55	3.00	1.80	7.00	-0.85	-0.05	3.95	4.25	-0.90	29.63	68.0	31.8	2.14
pf	+	0	—	None	2.80	3.50	3.80	4.85	4.75	0.50	1.40	-0.50	0.35	30.54	69.5	30.0	2.32
pg	+	0	—	None	2.95	3.95	2.80	5.65	4.50	0.55	0.75	-0.60	0.65	29.69	70.8	29.0	2.44
ph	+	0	—	None	2.75	4.35	4.00	5.40	4.30	0.95	0.70	-0.55	0.65	29.71	70.5	29.5	2.30
pr	+	0	—	None	3.30	5.10	2.40	4.10	4.55	1.25	1.15	-0.50	-1.10	29.74	69.1	30.8	2.24
rs	+	0	—	None	2.40	5.00	3.30	3.15	4.30	0.65	3.40	-0.80	-1.10	29.52	71.0	29.1	2.44
ru	+	0	—	None	3.20	4.00	3.70	3.35	3.30	1.00	2.00	-0.60	-1.20	28.87	70.2	29.6	2.37
sy	+	0	—	21"oxyf	3.40	5.25	1.90	2.45	4.70	0.15	3.25	-0.70	-1.15	30.75	67.0	32.4	2.08
ta	+	0	—	21"oxyf	3.35	5.00	2.90	2.95	4.85	1.25	1.15	-0.30	-1.15	29.77	69.0	30.9	2.24
tl	+	0	—	21"oxyf	2.45	4.85	2.00	5.45	4.55	0.55	-0.70	-1.10	29.59	68.8	30.9	2.23
Type E																	
pt	+	0	+	21"oxyf	2.82	5.10	3.75	5.35	-0.90	1.65	3.20	4.40	1.10	29.58	69.1	30.7	2.25
pu	+	0	+	21"oxyf	2.45	5.40	4.40	5.55	-0.90	2.45	3.30	4.95	0.65	29.00	67.0	33.2	2.02
pu	31.03	67.4	32.2	2.00
qs	+	0	+	19"oxyf	3.30	5.05	2.85	4.00	-0.95	2.35	1.75	4.50	1.15	28.01	67.9	31.8	2.14
qt	+	0	+	None	3.30	4.90	1.85	1.30	-0.95	3.25	3.45	4.70	2.30	26.73	71.0	28.9	2.46
rp	+	0	+	None	3.15	5.90	3.60	1.25	-0.60	1.00	3.15	4.50	0.95	30.29	67.8	31.8	2.13
po	+	0	+	19"oxyf	2.60	3.65	3.15	5.45	4.80	1.55	2.80	4.45	2.35	30.80	69.0	30.9	2.23
rt	+	0	+	19"oxyf	3.00	6.45	2.65	2.10	2.40	3.70	3.50	5.55	1.15	29.21	69.9	29.9	2.24
qy	+	0	+	None	2.80	5.35	2.70	4.70	4.80	0.80	3.35	5.25	-1.45	30.16	68.2	31.5	2.17
Type F																	
se	+	0	—	19"oxyf	-0.10	0.80	0.00	0.00	0.00	1.30	0.80	-0.90	-1.25	31.95	67.3	32.4	2.08
sg	+	0	—	19"oxyf	-0.70	0.40	0.00	-0.40	0.00	0.25	0.85	0.00	-0.35	31.15	66.0	33.8	1.95
sh	+	0	—	21"oxyf	-0.85	0.55	0.00	-0.45	0.00	0.35	0.85	0.00	-1.50	29.31	70.0	29.7	2.36
si	+	0	—	19"oxyf	-0.40	0.50	0.00	-0.45	1.05	0.95	0.85	-0.85	-1.00	31.40	66.0	33.3	1.98
sn	+	0	—	19"oxyf	-0.70	0.20	0.00	-0.30	0.00	1.30	0.85	-0.95	-1.15	31.95	67.8	32.0	2.12
so	+	0	—	-0.70	0.20	0.00	-0.30	0.00	0.55	1.25	-1.00	-1.50	31.82	67.9	31.9	2.12
sp	+	0	—	19"oxyf	-0.90	0.30	0.00	-0.15	0.00	0.05	0.90	-1.00	-1.50	32.31	66.1	33.6	1.97
sq	+	0	—	19"oxyf	-0.25	1.00	0.00	-0.40	-1.00	0.35	0.95	0.00	-1.50	32.75	66.1	33.7	1.96
sr	+	0	—	19"oxyf	-0.60	0.20	0.00	-0.25	0.00	0.75	1.05	-0.60	-1.40	31.70	66.7	33.0	2.02
ss	+	0	—	19"oxyf	-0.65	0.65	0.00	0.00	-0.45	1.60	1.15	-0.90	-1.15	31.03	67.9	31.9	2.13
uf	+	0	—	21"oxyf	-0.40	0.20	0.00	-0.20	0.00	0.30	0.60	0.00	-1.50	31.54	66.6	33.1	2.01
uh	+	0	—	19"oxyf	-0.45	0.00	0.00	0.00	0.00	1.10	0.85	-0.90	-1.40	32.00	66.3	33.4	1.98
uo	+	0	—	17"oxyf	-0.80	0.65	0.00	0.00	0.00	0.50	1.00	0.00	-1.15	31.15	68.1	31.3	2.18

fall the average results of the sixteen analyses of the gas produced by the eight low ratio cultures, as did those of 182 of the analyses of the gas produced by the 149 low ratio bacteria isolated from bovine feces. The average ratio in that series was $\text{CO}_2:\text{H}_2=1.06$, and in this series it was again 1.06.

In like manner, the absence of hydrogen in the gas produced by seven of the cultures characterized another group, shown in Chart 1 by the dots on the base line.

In the case of organisms giving the high ratios, the distinction from the other two groups was sharp, but the homogeneity within the group was not so clear. Similar high ratios with similar, though greater, variations were found in the analyses reported in our study of the gas-producing bacteria isolated from milk and milk products.¹⁴ We had hoped that improvements in our technic, especially the employment of a simpler medium, made with accuracy and without the usual vain attempts to correct its "reaction," would result in the appearance of

TABLE 4
AVERAGE VALUES IN GAS ANALYSES OF THREE GROUPS

Group	Total Gas, c.c.	CO ₂ c.c.	H ₂ c.c.	N ₂ c.c.	CO ₂ %	H ₂ %	N ₂ %	Ratio CO ₂ /H ₂
High ratio.....	29.50	20.67	8.75	0.08	70.1	29.7	0.2	2.36
Low ratio.....	12.41	6.37	6.00	0.04	51.3	48.3	0.4	1.06
∞ ratio.....	5.50	5.50	Trace	100 ?	Trace	∞

greater uniformity among the high ratios, as it did among the low ratios. This hope has thus far been dispelled, and we are forced to fall back upon the suggestion published in our first paper—that we have to do with two gas-producing reactions, one of which furnishes carbon dioxid alone. If this be true, then the end point of one reaction will have to depend strictly upon the other in order to avoid variations in the ratio $\text{CO}_2:\text{H}_2$ with small changes in conditions.

A few of the colon cultures isolated from cow feces which were described in our last paper, were carried for a time as usual on infusion agar and then a subculture of each was made in milk. These subcultures in milk were carried at 20 C. for thirty-three weeks with a transfer every seventh day. At the end of this period, gas determinations were made with these subcultures in a medium of the same composition that a year before had been used with the original cultures. The

14. Jour. Infect. Dis., 1914, 14, p. 411.

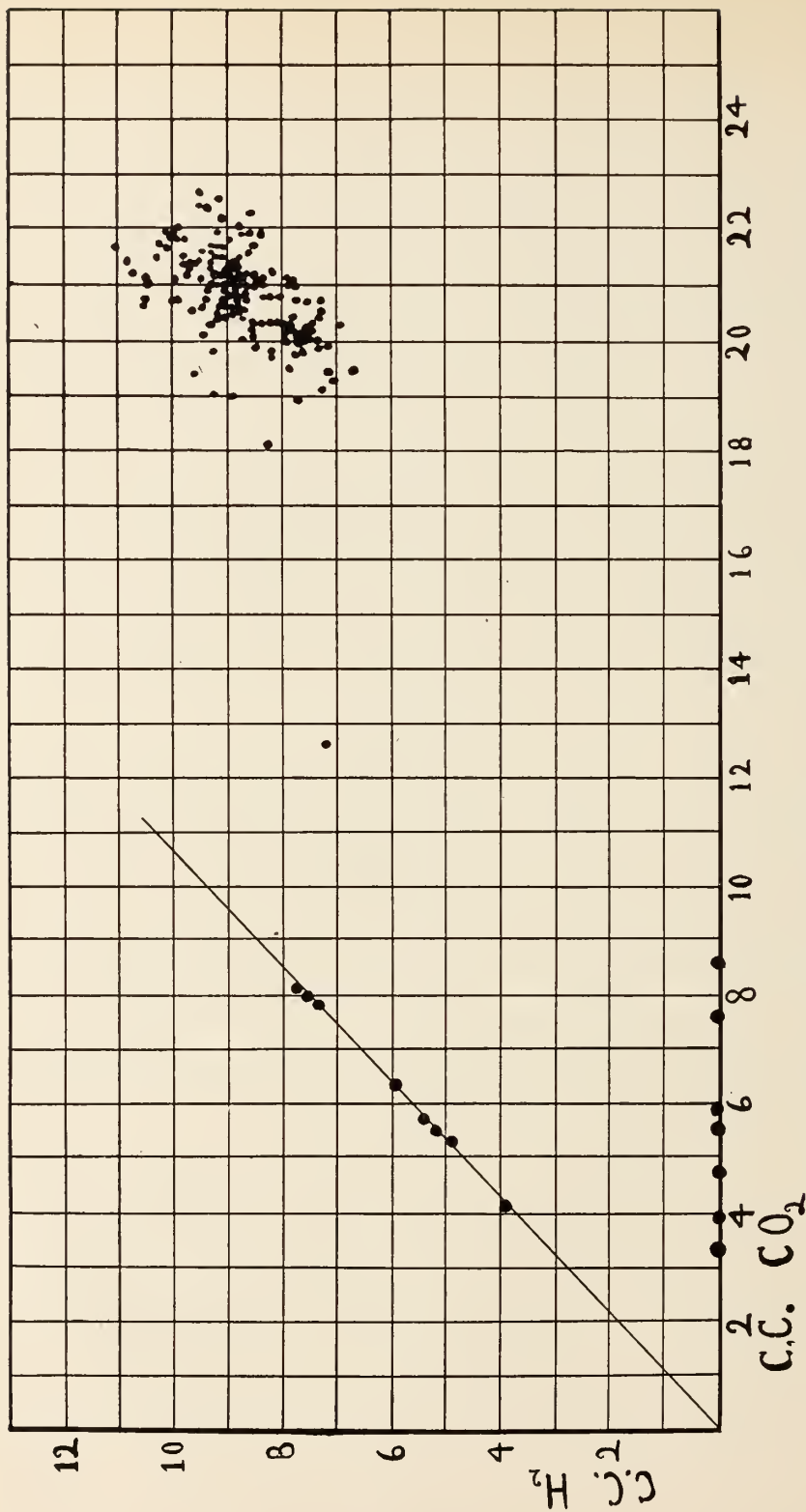


Chart 1.—Showing the differences in gas-production of the “high ratio,” “low ratio,” and ∞ groups of bacteria of the colon-aerogenes type.

gas determinations with the original young cultures and the old sub-cultures are given in Table 5. Their agreement speaks for itself.

CORRELATION OF RESULTS

The possibility of dividing this collection of cultures into three major groups on the basis of the carbon dioxid-hydrogen ratio has already been pointed out. The ability to bring about a certain fermentation with certain definite end products is evidently firmly fixed, and is more a mark of relationship than the ability to utilize any particular substance in bringing about this fermentation. In our earlier papers, it has been shown that the production of carbon dioxid and hydrogen in a certain definite proportion is closely correlated with other physiologic characteristics and is therefore a proper basis for subdivision.

TABLE 5
GAS PRODUCTION OF YOUNG AND OLD COLON CULTURES

Date	Culture	Total Gas, c.c.	CO ₂ %	H ₂ %	Ratio CO ₂ /H ₂
1913	jh.....	13.99	51.1	48.2	1.06
1914	14.10	51.4	48.1	1.07
1913	jm.....	11.64	51.3	48.7	1.05
1914	13.05	52.1	47.8	1.09
1913	jn.....	15.07	50.8	48.6	1.04
1914	16.09	52.1	47.7	1.09
1913	ma.....	13.42	51.6	47.8	1.08
1914	13.80	51.8	47.7	1.09
1913	nh.....	16.22	53.0	46.8	1.13
1914	14.41	51.9	48.1	1.08

One of the divisions of this collection, made on this basis, is separated radically from the other two groups by its failure to produce hydrogen. The general characteristics of this group have been brought together in Table 2, and summarized briefly in Table 8 (Type B).

This table shows that the cultures included in this group have many characteristics in common, in addition to the gas production. A capsule was observed on one culture only; all liquefied gelatin; only one produced indol from tryptophan; all fermented saccharose; only one fermented lactose or raffinose; glycerin was fermented by five of the seven and adonite by four, while none were able to utilize starch, inulin, or dulcitol. Nearly all the cultures produced decided pigment, but there was some variation in the color. The peculiar gas

production, the failure to ferment lactose, together with the liquefaction of gelatin, which in many cases is active, may be taken as sufficiently distinctive to separate these cultures from the colon group. However, any conclusions based on a study of seven cultures only must necessarily be tentative.

In our study of the colon cultures from bovine feces, it was found that they were almost exclusively of a type adhering very closely to the carbon dioxid-hydrogen ratio of 1.06. Eight of our grain cultures agreed with this type in that the gas ratio was identical. The characteristics of this group, which for convenience we will designate as Type A, are tabulated in Table 1 and summarized in Table 8.

TABLE 6
POSITIVE REACTIONS ARRANGED BY TYPES

Type	Cap- sule	Gela- tin	Indol	Saccha- rose	Lac- tose	Raffi- nose	Starch	Inu- lin	Man- nite	Glyce- rin	Ado- nite	Dul- cite
A	1* 12.5	0 0	4 50.0	2 25.0	8 100.0	6 75.0	0 0	1 12.5	8 100.0	2 25.0	3 37.5	4 50.0
B	1 14.3	7 100.0	1 14.3	7 100.0	1 14.3	1 14.3	0 0	0 0	7 100.0	5 71.4	4 57.1	0 0
C	0 0	40 100.0	0 0	40.0 100.0	40 100.0	40 100.0	0 0	0 0	39 97.5	32 80.0	0 0	40 100.0
D	32 30.5	0 0	0 0	90 100.0	90 100.0	87 96.4	19 21.1	9 10.0	11 12.2	51 56.6	6 6.7	13 14.4
E	8 100.0	0 0	8 100.0	8 100.0	8 100.0	8 100.0	8 100.0	3 37.5	7 87.5	8 100.0	8 100.0	5 62.5
F	13 100.0	0 0	0 0	0 0	13 100.0	0 0	0 0	0 0	5 38.4	4 30.8	0 0	0 0

* The first number = number of cultures; the second, percentage of total.

In a general way, the fermentation tests agreed also; but half of these cultures failed to produce indol, while the fecal organisms gave a positive reaction without exception. The difference in the pigment formation was even more marked, as is shown in Table 7. Six of the eight fall on the pure color of the 19" yoy plate, while one is classed as one tint lighter, and one as one shade darker. The decided yellow color of these cultures was readily noticeable, even in the comparatively young culture. This is in marked contrast to the colorless cultures of fecal origin.

These two groups, Types A and B, altho they were sharply defined, were numerically insignificant, making together only 15 of the 166 cultures. The remaining 151 cultures may be thrown together into a

group with gas ratios varying from 1.91 to 3.00. While it is evident that this is a heterogeneous group, there is no evidence of lines of separation on the basis of gas ratios as they are arranged in Chart 1.

We may make a tentative division, however, by grouping together the forty feeble liquefiers. Reference to Table 6 shows that these cultures, which we have designated Type C, all fermented saccharose, lactose, raffinose, and dulcitol, and, with one exception, all fermented mannitol. They failed completely to form a capsule, produce indol, or ferment starch, inulin, or adonitol. There was a very close agreement among themselves and in some respects a sharp differentiation from the other types. This is perhaps shown more clearly in Chart 2, which is a graphic representation of Table 6.

The type represented under D was the most numerous of the collection, including ninety cultures. It was distinguished from Type C, not only by the gelatin reaction, but also by the failure to ferment mannitol and dulcitol and, to a lesser extent, by the action on glycerin. A capsule was observed on 30 percent of the D cultures against none in the C type.

There remain two small groups, E and F, represented by eight and thirteen cultures, respectively. The activity of the E type is well represented in Chart 2, which shows nearly all the reactions, with the exception of gelatin, on the positive side of the line. A considerable proportion of the cultures fermented even the more resistant inulin. On the other hand, the F type failed almost completely on all of the tests with the exception of lactose. It should be observed however that some of these reactions might have been obscured by the secondary alkaline fermentation. A capsule was observed in all the cultures included in these two groups.

On the basis of the tentative grouping shown in Table 6 and Chart 2, frequency polygons have been constructed showing the distribution of cultures included in the high ratio types, C, D, E, and F, on the basis of the carbon dioxid-hydrogen ratio. This is shown in Chart 3. Altho the types overlap one another to some extent, each is confined to a definite part of the figure. The extreme low side is occupied by Type F, while, partly overlapping it, but distinctly higher, is Type E. On the extreme high side is Type C, while the center is occupied almost exclusively by Type D. This additional evidence of relationship strengthens very materially the tentative grouping made on the basis of the fermentation reactions more commonly employed.

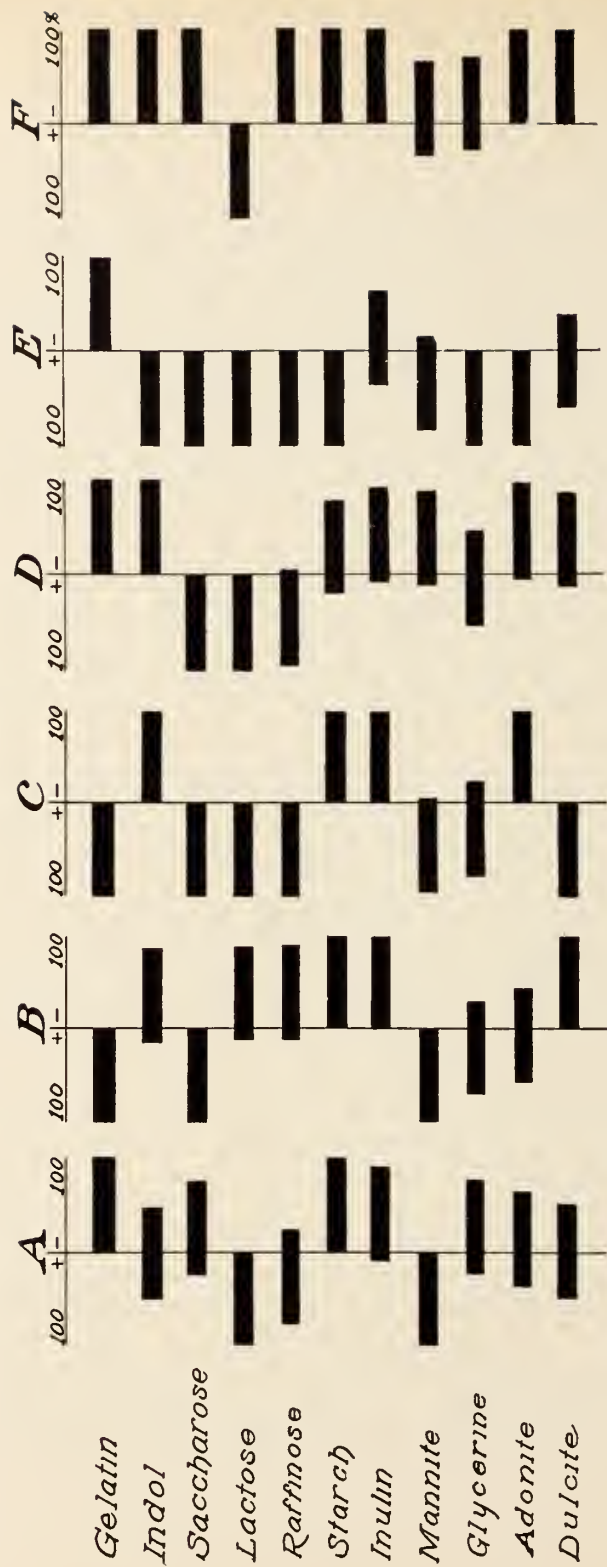


Chart 2.—Showing the differences in fermentation reaction of the A, B, C, D, E, and F types of the colon-aerogenes group. A positive reaction is plotted to the left, a negative one to the right of the median lines.

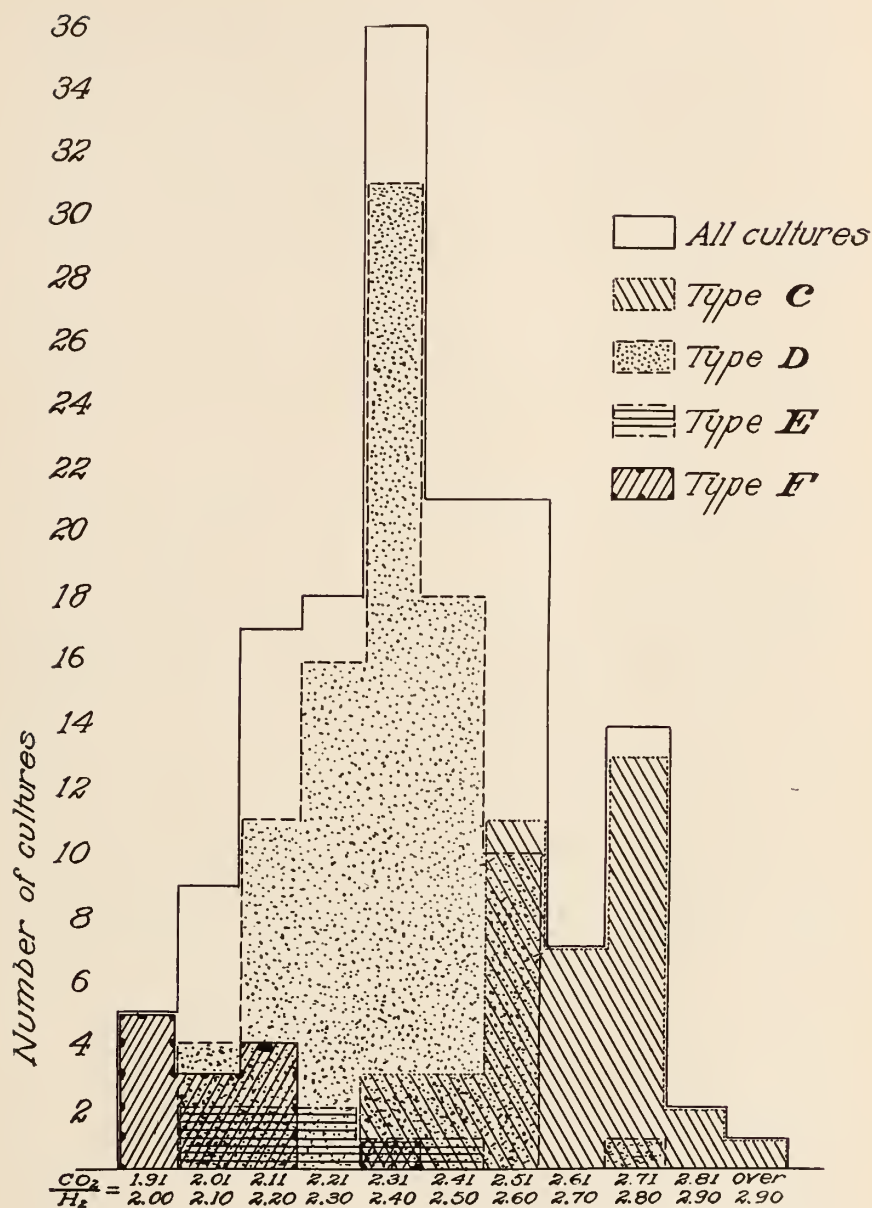


Chart 3.—Frequency polygons showing the distribution of cultures included in the high ratio types, C, D, E, and F on the basis of the carbon dioxide-hydrogen ratio.

In the previous work on the colon-aerogenes group, the formation of pigment was not considered a factor in the classification. This may have been because the types usually studied are nearly, or quite, free from pigment. Even a casual examination of the grain series showed that a large proportion of them were pigment formers to some degree. Usually this was so slight that the culture would be described as white; but when it was grown for some time at a low temperature and the growth spread on white paper, it was very easy to match these with standard colors. The results of these comparisons are tabulated in Table 7.

The degree of correlation in the pigment formation was greater than would appear at first glance because of the difficulty in arranging the table to bring together those colors most likely to be mistaken for

TABLE 7
DISTRIBUTION OF CULTURES BY PIGMENT FORMATION
Numbers and letters in heads refer to plates in Ridgeway's Color Standards and Nomenclature.

	No Pig- ment	17oy	17'oy	17"oy	19yoy	19'yoy				19"yoy				21"oyy	
		Cad- mium Yel- low	f Light Buff	d Pink- ish Buff	b Apri- cot Yel- low	Light Cad- mium	f Cream Color	d Naples Yel- low	b Mus- tard Yel- low	Prim- ulin Yel- low	f Cart- ridge Buff	b Cham- ois	i Honey Yel- low	i Isa- bella Color	f Ivory Yel- low
Type A....	1	6	1
Type B....	..	2	1	2	..	1	1
Type C....	6	20	..	7	7
Type D....	13	..	2	62	1	1	7	1
Type E....	3	3	2	..
Type F....	1	7	2	3	..

one another. The groups A, B, and C were distinctly pigmented, so much so that it would have been noticeable in the ordinary agar culture. Types D, E, and F, on the other hand, had only a small amount of pigment and would usually have been considered white. The A type, which is the one with the colon ratio, had six of the eight cultures under the pure color of 19" yoy, while one culture varied from it by one shade, and one by one tint. The seven cultures of Type B were somewhat scattered, but of the forty cultures of Type C, twenty agreed with the pure color of 19 yoy, six differed from this by one shade, and fourteen were matched with colors which differed from these two by the addition of a small amount of neutral gray. The D cultures had only a small amount of pigment, practically all falling in the f-tints, which are those nearest the white. It is not easy for the ordinary eye to distinguish between the light-buff, cream-color, car-

tridge-buff, and ivory-yellow with which the cultures of this type were matched. Types E and F followed D very closely.

Table 8 is arranged to show the reactions of the typical culture of each of the six types. In Types A, E, and F, the number of cultures examined was not large enough to establish a type with any finality, but the characteristics of B were so radically different that there is no question about its separation from the others. Type C, with its forty cultures agreeing with one another almost like duplicates, may be said to be well established. The large group, D, is composed of ninety cultures with a relatively small number of variants. In comparing these types with those in other collections, it should be

TABLE 8
REACTIONS OF TYPE CULTURES

	A	B	C	D	E	F
Ratio $\frac{\text{CO}_2}{\text{H}_2}$	1.06	∞	2.51-2.80	2.20-2.50	2.10-2.30	1.90-2.20
Pigment.....	Honey yellow	Cadmium yellow, pinkish buff	Light cadmium	Cream color	Cream color	Cream color
Capsule.....	—	—	—	—	+	+
Indol from tryptophan....	±	—	—	—	+	—
Gelatin.....	—	+	+	—	—	—
Saccharose.....	—	+	+	+	+	—
Lactose.....	+	—	+	+	+	+
Raffinose.....	+	—	+	+	+	—
Starch.....	—	—	—	—	+	—
Inulin.....	—	—	—	—	±	—
Mannite.....	+	+	+	—	+	±
Glycerin.....	±	+	—	±	+	±
Adonite.....	±	+	—	—	+	—
Dulcitate.....	±	—	+	—	+	—

remembered that the gas ratio may vary with conditions and that gas determinations made with Smith tubes are not comparable with those made by Keyes's method.

CONCLUSIONS

By careful selections of the manifestations of the physiologic activities most characteristic of a particular group of bacteria, it is possible, by the collection of sufficiently accurate data, to bring together the closely related cultures into natural species, or varieties, and to establish lines of separation as sharply defined as is possible in view of the overlapping which actually exists in nature. The stability of this grouping cannot be shaken by evidence of variation in some of the characteristics used for purposes of classification. The fact that

these cultures with their peculiar grouping of characteristics occur in large numbers in similar habitats in widely separated localities is almost conclusive evidence that the groups into which they fall are valid. Species so established cannot be identified or separated from one another by the turn of a single characteristic. Natural groups of bacteria are bound together by the common possession of a certain combination of characteristics, no one of which is absolutely fixed. To attempt to separate a family into species by dividing all those that respond to some arbitrary test from those that fail to respond, is certain, in many cases, to remove a culture from its natural relatives. It may seem that we have violated this principle in making primary separations on the basis of the gas ratio. It should be remembered however that this is only one of numerous correlated characteristics which separate the major group into distinct minor groups.

Within the limits of the colon-aerogenes group, there are several well-defined sub-groups, or species. One of these is peculiar to the digestive tract, or at least to the bovine digestive tract, and is characterized by the relatively low gas production, a carbon dioxid-hydrogen ratio under definite conditions of 1.06, the uniform production of indol from tryptophan, and the fermentation of dulcitol.

It would not be safe to say that the bacteria discussed in this paper had their normal habitat on grains and on grains exclusively. The method of collection, with the possible exception of the green oats, did not exclude the possibility of contamination, and the fact that the grains were thoroughly dried has some bearing on the question. Against the supposition that these bacteria were mere accidental contaminations carried by the air dust and probably of fecal origin, was the very uniform and marked difference between the grain types and the bovine type. It should also be noted that of the fifteen cultures obtained from two samples of green oats, four belonged in Type B, one in Type C, and ten in Type D.

It is significant that the same definite type of bacteria should be found on grains coming from so many widely separated sources and that this type should differ in so many respects from that found in bovine feces.

Of the six types into which this collection of cultures has been divided, three may be considered well established. One of these, B, occurred only a few times; but on account of its peculiar gaseous fermentation, its failure to ferment lactose, and the liquefaction of gela-

tin, it may be considered as a distinct variety which has at best only a slight connection with the colon-aerogenes group. Notwithstanding this marked difference, certain lactose-fermenting strains may easily be mistaken for *Bacillus coli*. Indeed, one of the cultures furnished us by Mr. Kligler as an atypical colon belonged in this group.

The validity of including the group designated as Type C in the colon-aerogenes group may be questioned. In the classification of the colon group adopted by the Laboratory section of the American Public Health Association,¹⁵ failure to liquefy gelatin in fourteen days is given as a characteristic of the *Bacillus coli* group, altho one variety is given which liquefies gelatin in twenty days. So far as published descriptions permit us to make comparisons, this type agrees with *Bacillus cloacae*. Jordan,¹⁶ who first described this organism, considered that it belonged to the proteous group, but many later writers seem inclined to place it with the colon-aerogenes group. In any case, the cultures which we have included in this group are typical of the slow liquefiers, which may be included in the colon-aerogenes group without question. The gelatin liquefaction is so slow and the pigment production so slight that these characters could easily be overlooked. It is not improbable that they may, at times, be entirely lost.

The type D, if the number of cultures in this collection may be taken as an indication, is the most common of the grain varieties. It differs from C in gelatin liquefaction, and mannite and dulcitate fermentation, and from E in indol production, and starch, mannite, adonite, and dulcitate fermentation. In this group we find that something over one-half the cultures adhere very closely to the type. The remaining cultures may be classed as variants from the type. It should be noted however that these variations do not occur in single characteristics, but that two or three characteristics of a culture vary together. In some cases, for instance, the presence of a distinct capsule is correlated with an entire absence of pigment, a strong fermentation of starch and inulin, and a failure to ferment dulcitate. That there are usually several variants with identical characteristics may be due in some cases to the fact that they all came from the same sample, but it frequently happened that identical variants came from widely separated sources. The question of considering these as variants or as a distinct type, is largely a matter of opinion. With the information now available, we prefer to consider them as variations from the type. This type may

15. Standard Methods for the Examination of Water and Sewage, 1912, p. 80.

16. Jour. Hyg., 1903, 3, p. 1.

be identified perhaps with the ordinary conception of *Bacillus aerogenes*. Kligler describes *Bacillus aerogenes* as lactose +, saccharose +, salicin +, glycerin +, gelatin —, indol \mp , a description agreeing for the most part with our Type D, except that not one of our ninety cultures gave a positive test for indol. However, the particular name which we shall apply to these groups, or their identity with previously described species, is of no great importance at this time. The really significant fact established is the existence of clearly defined varieties, or sub-groups, of this family, and their occurrence in a definite habitat.

SUMMARY

The work of other investigators has shown that bacteria of the colon type occur frequently on grains, fruits, and grasses; but these are usually assumed to be identical with the fecal type, if not merely contamination with fecal matter carried as dust.

Our own investigations have demonstrated that the colon bacteria of the bovine intestine belong, apparently almost without exception, to a single, sharply defined type characterized by the production of hydrogen and carbon dioxid in the proportion of 1 to 1.06.

A collection was made of 166 cultures of colon-like bacteria obtained from thirty-three samples of dried grains coming from various states, and from two samples of green oats. On the basis of the gas produced in the anaerobic fermentation of dextrose, these cultures may be divided into three general groups:

Seven cultures produced hydrogen only. These cultures liquefied gelatin and did not produce indol. They usually fermented saccharose, mannite, glycerin, and sometimes adonite, but usually failed to ferment lactose, raffinose, starch, inulin, and dulcite. They formed a yellow pigment on agar.

Eight cultures gave a gas ratio of 1.06 in agreement with the fecal type, but differed in the failure of half the cultures to form indol, in their failure to ferment glycerin, and particularly in their production of a yellow pigment.

All of the remaining cultures gave gas ratios varying from 1.90 to 3.00.

Of these 151 cultures, forty liquefied gelatin slowly and fermented saccharose, lactose, raffinose, and dulcite. They formed a yellow pigment corresponding to the light cadmium of Ridgway's plates. Nearly all fermented mannite and glycerin. All failed to form indol and to

ferment starch, inulin, and adonite. The carbon dioxyd-hydrogen ratio varied from 2.30 to 3.00.

Ninety cultures constituted one type, of which all fermented saccharose, lactose, and raffinose and about one-half fermented glycerin. All failed to liquefy gelatin, and practically all failed to ferment starch, inulin, mannite, adonite, and dulcite. The carbon dioxyd-hydrogen ratio ranged from 2.00 to 2.60.

Eight cultures were grouped together by the formation of indol and particularly by their marked fermentative ability. Practically all the cultures fermented all the test substances used. The gas ratio was between 1.90 and 2.20.

Thirteen cultures differed from the other types in failing to liquefy gelatin, form indol, or to ferment any of the test substances except dextrose and lactose.

None of the types found on grains agreed with the characteristic flora of bovine feces. Even the type with corresponding gas ratio was easily distinguished by its pigment formation.

THE DIFFERENTIATION OF BACTERIA OF THE COLON-AEROGENES FAMILY BY THE USE OF INDICATORS *

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INTRODUCTION

It is a rare pleasure to see an old scientific deduction re-established after a period of abuse and rejection. Dr. Theobald Smith discerned in the differences of gas production among bacteria of the colon-aerogenes family a means of differentiation. That distinct differences exist has not been seriously disputed; but the methods of detecting them were left with no valuable improvements for nearly twenty years. During this time an enormous mass of inaccurate data accumulated and produced a confusion from which it was impossible to extricate conclusions sufficiently clean-cut to force conviction. Consequently, the gas ratio was rejected as untrustworthy, the Smith fermentation tube, after the exhaustion of its value as an instrument for pioneer research, was relegated to its proper position, and the more fundamental questions which its use had raised were to a large degree forgotten.

Then Keyes¹ pointed out the reasons for the hopeless inaccuracies of the fermentation tube and developed an accurate method for the collection of the gases. With slight improvements upon this method, Rogers, Clark, and Davis² found that they could obtain results which furnished an admirable basis for the differentiation of the colon-aerogenes group.

In the three investigations now completed by Rogers, Clark, and Davis² and Rogers, Clark, and Evans,³ the fundamental deduction of Theobald Smith upon the value of the gas ratio has been abundantly confirmed. It has been shown that bacteria of the colon-aerogenes family can be sharply divided into two groups on the basis of the gas ratio. When grown in vacuo in a medium consisting of 1 percent Witte peptone, 1 percent dextrose, and 0.5 percent K_2HPO_4 , one group produces

* Received for publication May 3, 1915.

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1. Jour. Med. Research, 1909, 21, p. 69.

2. Jour. Infect. Dis., 1914, 14, p. 411.

3. Ibid., 15, p. 99; Ibid., 1915, 17, p. 137.

carbon dioxid and hydrogen in the constant ratio $\text{CO}_2/\text{H}_2=1.06$. Members of this group have been termed "the low ratio organisms." The second group produces much more carbon dioxid than hydrogen, and furnishes a ratio which varies from about 1.90 to 3.00. Such bacteria are called members of "the high ratio group."

In the experimental work referred to, there were encountered a few organisms which produce carbon dioxid but no hydrogen. They are distinguished by certain cultural characteristics which set them apart from the colon-aerogenes family, but they will be treated in the following discussion as a third group. Since they liberate no hydrogen, the ratio $\text{CO}_2:\text{H}_2$ becomes infinity. We shall retain the term " ∞ ratio group" as a convenient means of designation.

Every advance made in the study of these three groups of gas-producing bacteria has added force to the belief that the gas ratio is an index to certain fundamental differences in metabolism. Consequently, when accurately determined, it may be depended upon to furnish a logical basis for the preliminary separation into main groups. Such use of it was made in the three investigations to which reference has been made. In the results there appeared a remarkable correlation between the gas ratio and the source of the cultures, which is summarized in the following table:

TABLE 1
CORRELATION BETWEEN GAS RATIO AND THE SOURCE OF THE CULTURE

Source	Total Number of Cultures	Number of Low Ratio Cultures	Number of High Ratio Cultures	Number of ∞ Ratio Cultures
Milk and milk products.	124	65	59	1
Bovine feces	150	149	1	0
Grain	166	8	151	7

The sanitary significance of this correlation is perfectly obvious. But it is also clear that the methods by which the gas ratio can be accurately determined are not adapted to the routine examination of large numbers of cultures. This leaves certain laboratories in a rather embarrassing position, for while the sanitary significance of the gas ratio may be appreciated, no method applicable to routine work is available. It is therefore imperative that some characteristics be found which will correlate accurately with the gas ratio, and which may be accurately and easily distinguished. By taking cognizance of certain

characteristics of the cultures at hand, the desired correlation has been found, and a simple method of diagnosis worked out. This method can best be presented by showing its logical development.

DEVELOPMENT OF THE METHOD

In 1912 Michaelis and Marcora⁴ showed that a certain culture of *Bacillus coli*, when grown in a lactose broth, ceased activity at a hydrogen ion concentration of 1×10^{-5} N (this term being an abbreviation for the equivalent of 0.00001 gm. H per liter). This point is reached independently of the initial reaction of the medium. Michaelis and Marcora considered it to be a physiologic constant for *Bacillus coli*.

Since these authors apparently worked with but one culture and one medium, it remained to be determined whether this limiting hydrogen ion concentration is a constant for other cultures of *Bacillus coli* and in media of such widely varying composition that it could truly be called a characteristic physiologic constant. Such a research has been progressing for two years and during this time so many interesting facts have been observed that publication has continuously been delayed pending "the next set of experiments." In view of the fact that certain of the results have proved to be of immediate practical value in solving the problem at hand, they may be sketched as follows:

All organisms of the colon-aerogenes family which give a low gas ratio will, if furnished sufficient fermentable carbohydrate, continue to elaborate acid until a certain zone of hydrogen ion concentration is reached. There all activity ceases. The particular point in this zone is determined by the nature of the medium; but in any given medium the particular point reached by a variety of cultures is remarkably constant. In a medium such as that used by Michaelis and Marcora, the point is very close to 1×10^{-5} , as these authors found. With simpler media, containing dextrose, the point reached is appreciably higher, as Table 2 will show.

TABLE 2
FINAL HYDROGEN ION CONCENTRATION OF "LOW RATIO CULTURES"

Organism	CH ⁺
22 fg	4.8×10^{-5}
22 fg	4.7×10^{-5}
22 hw	4.9×10^{-5}
22 hw	5.0×10^{-5}
22 hx	4.8×10^{-5}
22 hy	4.8×10^{-5}
22 hy	5.1×10^{-5}
22 hz	4.7×10^{-5}
22 hz	4.9×10^{-5}

* The medium consisted of 1 percent Witte peptone and 1 percent dextrose in Jena test tubes. Incubation was maintained at 30 C. for a period of fifteen days.

In determining hydrogen ion concentrations, the electrometric method has been used. The chain employed was the customary one: Hg HgCl | N/10 KCl | N/10 KCl | Sat. KCl | Solution | H₂ Pt. The electrode vessel was a special form based upon the principles of Hasselbalch's⁵ improved design. This and other details will be described elsewhere.

On the other hand, cultures of the "high ratio organisms," in media in which the "low ratio organisms" reach their limiting hydrogen ion concentration, are found to reach much lower values. This is shown in Table 3. The gas ratios included in this table are those given in the papers of Rogers, Clark and Evans.³

4. Ztschr. f. Immunitätsforschung, 1912, 14, p. 170.

5. Biochem. Ztschr., 1913, 49, p. 433.

TABLE 3

HYDROGEN ION CONCENTRATIONS OF "LOW" AND "HIGH RATIO CULTURES"* COMPARED

Organism	Ratio CO ₂ : H ₂	CH ⁺	Organism	Ratio CO ₂ : H ₂	CH ⁺
22 hx	1.03	2.8×10^{-5}	22 ty	2.60	7.3×10^{-7}
22 hz	1.06	3.3×10^{-5}	22 se	2.59	9.0×10^{-7}
			22 se	2.08	6.7×10^{-7}
22 om	2.28	6.0×10^{-7}	22 nd	2.22	6.3×10^{-7}
22 hg	2.44	5.1×10^{-7}	22 ni	2.52	1.0×10^{-7}
22 rq	2.36	7.0×10^{-7}	22 nn	2.24	5.7×10^{-7}
22 rr	2.24	7.0×10^{-7}	22 no	2.18	5.7×10^{-7}
22 rs	2.44	7.0×10^{-7}			
22 rt	2.34	1.6×10^{-6}			

* The medium consisted of 1 percent Witte peptone and 1 percent dextrose, 0.050 Molecular H₃PO₄; 0.075 Molecular Na OH. The incubation temperature was 30 C. for a period of seven days. Cultures were held in Jena test tubes.

It must not be inferred from this that the "high ratio organisms" cease fermentation at such low hydrogen ion concentrations because they can endure none higher. Rather does it appear that these organisms have fermented all the sugar with the production of a quantity of acid insufficient to inhibit further activity. If the dextrose content of the medium be increased, it is found that, up to a certain concentration of sugar, the gas produced is increased and the final hydrogen ion concentration is raised. This is shown in Table 4.

TABLE 4

GAS PRODUCTION OF "HIGH RATIO ORGANISMS"*

Organism	Percentage of Sugar	Total Gas in c.c.	Ratio CO ₂ : H ₂	Final CH ⁺ of Evacuated Medium
22 on	0.5	15.58	1.83	2.1×10^{-7}
	1.0	31.09	2.28
	2.0	54.93	3.04	2.9×10^{-7}
	4.0	66.35	2.92	1.2×10^{-5}
	8.0	71.37	3.00	8.0×10^{-6}
22 pb	0.5	16.21	1.70	3.1×10^{-7}
	1.0	30.98	2.23	3.1×10^{-7}
	2.0	57.13	2.85	3.2×10^{-7}
	4.0	72.15	2.88	6.8×10^{-6}
	8.0	83.31	2.98	1.1×10^{-5}
22 qt	0.5	15.31	1.62	3.1×10^{-7}
	1.0	26.73	2.46	4.7×10^{-7}
	2.0	48.81	2.69	1.4×10^{-5}
	4.0	49.92	2.71	2.4×10^{-5}
	8.0	47.53	2.82	3.0×10^{-5}
22 rh	0.5	Lost	1.9×10^{-7}
	1.0	27.86	2.60	5.3×10^{-7}
	2.0	54.40	2.81	6.2×10^{-7}
	4.0	49.34	3.09	1.7×10^{-5}
	8.0	51.96	3.15	1.7×10^{-5}

* The medium consisted of 1 percent peptone and 0.5 percent K₂HPO₄ plus different concentrations of dextrose (10 c.c. of medium in vacuum bulbs). Incubation was maintained at 30 C.

From this experiment, it is evident that the low values of the hydrogen ion concentrations found in dilute sugar cultures of the "high ratio organisms" are not limiting values. What the limits are is yet to be accurately determined.

For the present purposes, we need not know them. The only point with which we now are concerned is that in dextrose concentrations of 1 percent or lower, and in the presence of sufficient protein, dibasic phosphate, or similar "regulator," the limiting hydrogen ion concentration is not reached in "high ratio cultures."

The next point to decide is how far we may lower the sugar content and still allow a sufficient amount for the attainment of maximal acidity in "low ratio cultures." The exact minimum we have not determined, but it will appear later that 0.5 percent dextrose in a medium containing 0.5 percent peptone and 0.5 percent K_2HPO_4 is safely above the minimum.

The next point to which attention must be called is this: If an organism of either class be cultivated in a medium containing sufficient "regulator" to prevent a great rise in hydrogen ion concentration with small increases in the acid content, and if the sugar content is then so low that the limiting hydrogen ion concentration cannot be reached, the medium under aerobic conditions will become more alkaline after the exhaustion of the sugar.

This is shown admirably in Chart 1. In this experiment, a "low ratio organism" (22hx) was cultivated at 37.5 C. in media containing 1 percent Witte peptone and varying percentages of dextrose. The cultures were held in Jena test tubes. From time to time a tube of each of the six media was cooled to 30 C. and its hydrogen ion concentration measured electrometrically. In plotting the results, the hydrogen ion concentrations have been laid off as ordinates against days as abscissae.

Similar results are obtained in other media with both high and low ratio organisms, but, with a high ratio culture, in order to prevent a reversion of the reaction, the dextrose content must be raised considerably above 1 percent.

As a result of these experiments, we concluded that in a medium consisting of 0.5 percent Witte peptone, 0.5 percent dextrose, and 0.5 percent K_2HPO_4 , the "low ratio cultures" would attain their maximal hydrogen ion concentration without a reversion; while the "high ratio organisms" would not attain a limiting hydrogen ion concentration and consequently there would be a tendency to reversion of the reaction. That such is the case is shown in Chart 2. In this experiment, two "high ratio organisms" (pb and qt), one "low ratio organism" (hx), and one "∞ratio organism" (tj) were grown aerobically in Jena test tubes at 30 C. in media made up of 0.5 percent peptone, 0.5 percent dextrose, and 0.5 percent K_2HPO_4 . The hydrogen ion concentration (ordinates) is plotted in Chart 2 against hours (abscissae). Between the twenty-eighth and fiftieth hours, the "low" and "∞ratio cultures" were still rising, while the "high ratio cultures" had begun to decline. Then from the fiftieth hour all but the "low ratio culture" declined. The "low ratio culture" had shown no signs of a reversion after 288 hours. The decline in hydrogen ion concentration is not due to loss of carbon dioxide, for the measurements were made after evacuation of the cultures with a Geryck pump. Nor should it be assumed that the reversion is due solely to an ammonia production. Rather does it appear to be due to another well-defined phenomenon, which we shall consider in another paper.

From all these experiments and several others, which it is not essential to describe, we are in a position to make a good guess that, in a medium consisting of 0.5 percent peptone, 0.5 percent dextrose, and 0.5 percent K_2HPO_4 , all "low ratio cultures" at the end of 3 to 4 days will show their limiting hydrogen ion concentrations, while all

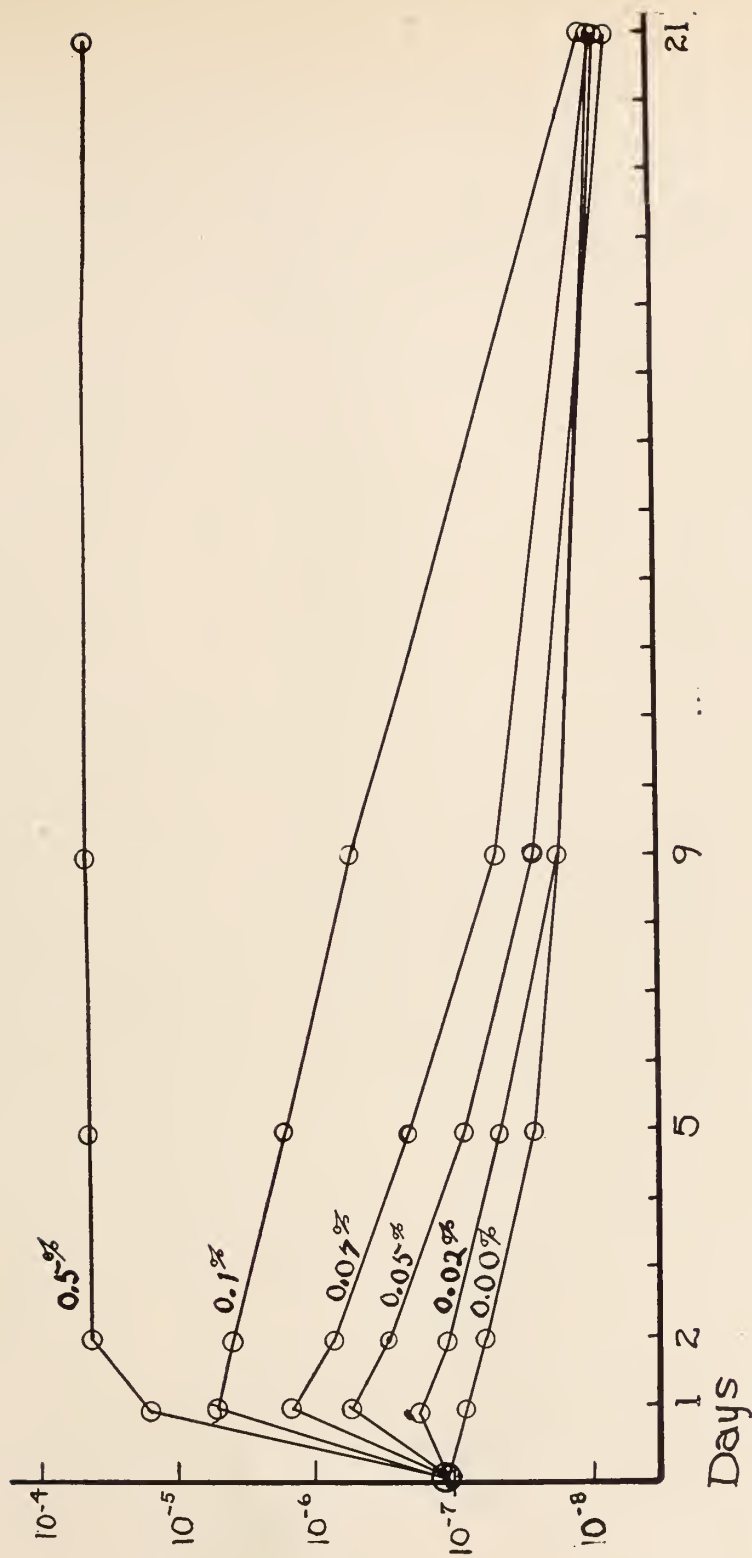


Chart 1.—Progressive change in hydrogen ion concentrations in 1 percent peptone + different percentages of dextrose. A low ratio culture (22 hx) at 30 C.

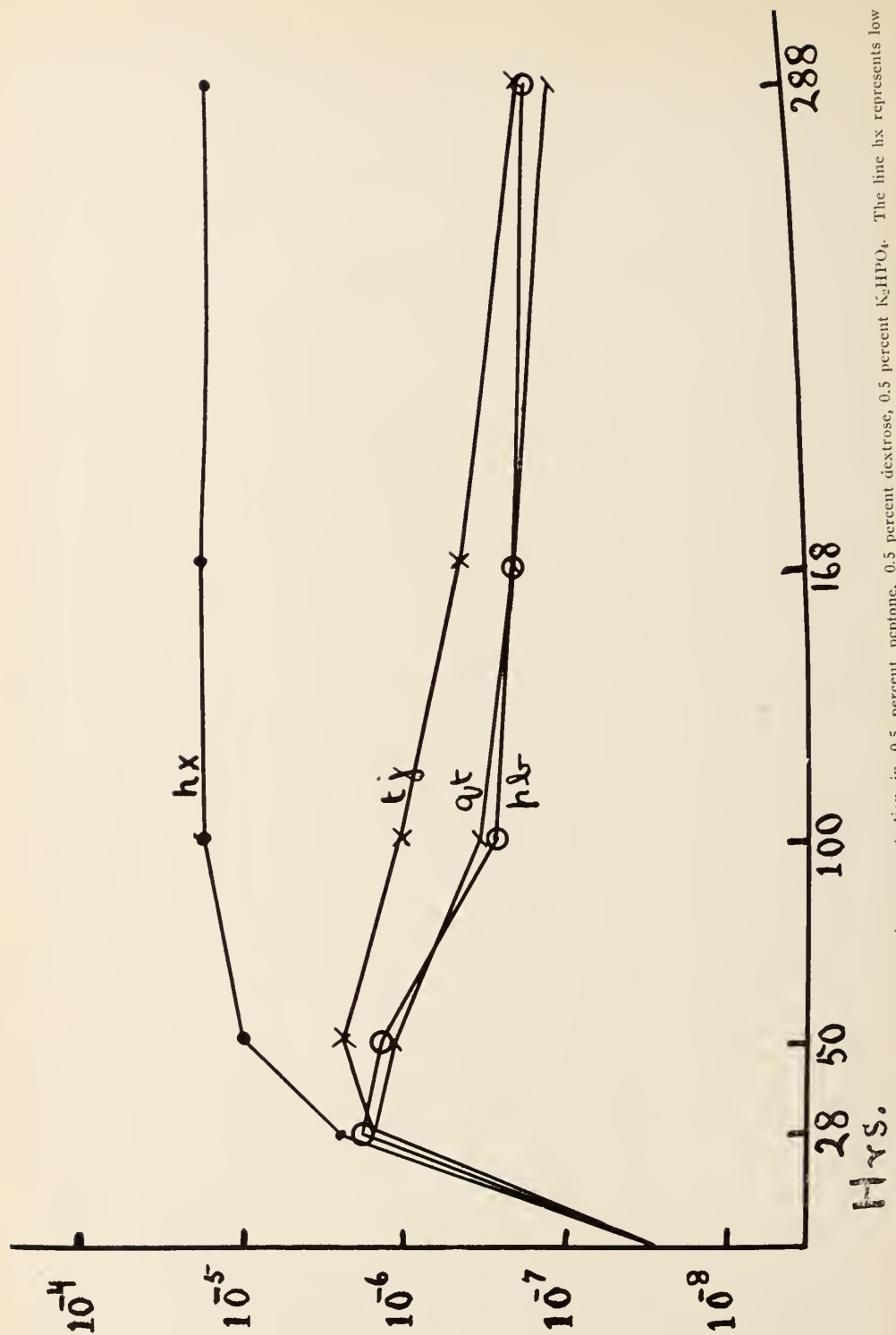


Chart 2.—Progressive change of hydrogen ion concentration in 0.5 percent peptone, 0.5 percent dextrose, 0.5 percent K_2HPO_4 . The line hx represents low ratio culture; u, ∞ ratio culture; qt and pb, high ratio cultures.

"high ratio cultures" will show a hydrogen ion concentration far below this. Even in media less favorable for the detection of such differences, as we have already shown in Table 3, the differences detected by electrometric methods are great. Some results with the "0.5 percent medium" (p-) are shown in Table 5.

TABLE 5
HYDROGEN ION CONCENTRATIONS OF LOW AND HIGH RATIO CULTURES*

Culture	Ratio	CH ⁺	Cultures	Ratio CO ₂ : H ₂	CH ⁺
hx	1.03	2.0×10^{-5}	qw	2.23	1.0×10^{-7}
st	1.06	1.0×10^{-5}	rr	2.24	2.2×10^{-7}
oo	2.39	3.6×10^{-7}	rs	2.44	5.4×10^{-7}
pb	2.23	5.9×10^{-7}	ru	2.37	2.0×10^{-7}
pg	2.44	5.1×10^{-7}	rx	2.50	5.1×10^{-7}
pj	2.92	1.3×10^{-7}	ry	2.60	7.3×10^{-7}
po	2.23	7.8×10^{-7}	sa	2.78	5.1×10^{-7}
pw	2.39	0.1×10^{-7}	sd	2.08	2.3×10^{-7}
py	2.36	3.5×10^{-7}	so	2.12	1.5×10^{-7}
qb	2.42	0.5×10^{-7}	tc	2.37	4.3×10^{-7}
qr	2.34	0.7×10^{-7}	tu	2.82	4.4×10^{-7}

* The medium consisted of 0.5 percent Witte peptone, 0.5 percent dextrose, and 0.5 percent K₂HPO₄ in Jena test tubes (aerobic growth). Incubation was maintained at 30 C. for a period of 3-5 days.

To detect differences of such magnitude, it is not necessary to use the hydrogen electrode. The well-known colorimetric method is applicable, even without the use of comparison solutions of known hydrogen ion concentration.

In selecting a proper indicator for this purpose, we must choose one which, unlike the majority of indicators, will give correct indications of hydrogen ion, concentration in the presence of peptone. Two such indicators which may be used in the zone of hydrogen ion concentration with which we are concerned have already been studied. They are paranitrophenol, which Sørensen⁶ has used with good effect, and methyl red, which Palitzsch⁷ found to be reliable.

Paranitrophenol exists as a colorless compound in solutions with a hydrogen ion concentration above 1×10^{-5} N. In solutions of lower hydrogen ion concentration, it gradually becomes colored until at 1×10^{-6} it is a brilliant yellow green. By the use of comparison solutions of known hydrogen ion concentration, it is possible to make a very accurate estimation of the hydrogen ion value of a medium if it falls within this zone. This more exact method has not been found necessary in our procedure.

In the same way, methyl red is useful in approximately the same zone and its color changes are much more varied and brilliant, ranging from a brilliant red above 1×10^{-5} to a clear yellow below 1×10^{-6} . The color changes are so brilliant that we recommend this indicator in preference to paranitrophenol.

CULTURES TESTED

Before proceeding to a description of the results obtained with the indicator test, it will be well to classify the cultures used.

6. Compt. rend. trav. de Lab. Carlsberg, 1909, 8, p. 1.

7. Ibid., 1911, 10, p. 162.

Of the organisms isolated from milk and milk products and described in the first paper by Rogers, Clark, and Davis,² there were available only 22 s and 22 aj, both "high ratio cultures." In this same paper, 22 fg, a "low ratio organism" from human feces, was described. This was available.

Of the organisms isolated from bovine feces and described by Rogers, Clark, and Evans,³ there were available seventeen of the "low ratio cultures," and the one "high ratio culture."

Of the cultures isolated from grain and described by Rogers, Clark, and Evans in their second paper,³ there were 155 "high ratio cultures," 8 "low ratio cultures," and 7 " ∞ ratio cultures." All were available. We had also another "low ratio organism." Besides these cultures, we had six sent to this laboratory by Dr. Kligler and described as atypical members of the colon family. The gas determinations with these cultures are given in Table 6. The methods and special medium employed were those described in the papers by Rogers, Clark, and Evans.³

TABLE 6
GAS DETERMINATIONS WITH KLIGLER'S "ATYPICAL CULTURES"

Kligler's Number	Dairy Division Designation	Total Gas in c.c.	Ratio CO ₂ : H ₂
451	22 ol	27.18	2.40
452	22 oh	16.72	1.47
453	22 ok	6.87	1.06
454	22 of	18.27	2.28
179	22 og	0.91	CO ₂ and H ₂ present
370	22 oj	8.02	∞

So far as gas analyses indicate, three of these cultures agree very well with typical cultures of our own collection. This can best be shown by tabulating the data under consideration with the averages of our own cultures as is done in Table 7.

TABLE 7
COMPARISON OF GAS DETERMINATIONS IN ATYPICAL CULTURES WITH TYPICAL CULTURES

Organism	Total Gas c.c.	Ratio CO ₂ : H ₂
451 — 22 ol	27.18	2.40
Average of high ratio cultures, grain series.....	29.50	2.36
453 — 22 ok	6.87	1.06
Average of low ratio cultures, feces series.....	14.09	1.06
Average of low ratio cultures, grain series.....	12.41	1.06
370 — 22 oj	8.02	∞
Average of ∞ ratio cultures, grain series.....	5.50	∞

If, then, we classify these three of Kligler's six cultures as typical, it leaves in our collection three which may be described provisionally as atypical. We understand that Kligler's cultures were from the collection of the American Museum and had probably been carried upon artificial media for a long time.

In summary, we had 20 "low ratio cultures," 159 "high ratio cultures," 8 " ∞ ratio cultures," and the three "atypical" strains, which we have not yet extensively studied.

PRELIMINARY TESTS

When the time arrived for the gas determinations with the grain cultures, enough was known of the basis of the method which we are here describing to induce us to test the medium after the collection of the gas. In every case, the "low ratio cultures" were acid to paranitrophenol, and the "high ratio cultures" alkaline. All the " ∞ ratio cultures" in this experiment were acid.

To see whether the same differentiation would hold under the simpler aerobic conditions of test tube culture, we tested all these grain cultures again. In this experiment, the medium was that employed in the gas determinations, except that the peptone was lowered to 0.5 percent to gain a solution of less color. Again all the "low ratio cultures" at the end of a week were acid to paranitrophenol, while all the "high ratio cultures" were distinctly alkaline, except four. Of the " ∞ ratio organisms," three were distinctly alkaline, one distinctly acid, and three doubtful. As we shall see, the modified medium, developed for the final tests, eliminated the exceptions among the high ratio organisms.

FINAL TESTS

With the medium modified in accordance with the principles discovered in developing the method, we tested all the cultures available. Since the methods used are those we can confidently recommend, they will be described in detail.

Medium.—0.5 percent Witte peptone, 0.5 percent dextrose, 0.5 percent K_2HPO_4 . To prepare 1,000 c.c. of this, mix 5 gm. Witte peptone, 5 gm. chemically pure dextrose, and 5 gm. of K_2HPO_4 in 800 c.c. distilled water. Heat with occasional stirring for twenty minutes over steam and filter through a Schleicher and Schüll No. 588 folded filter. Cool to 20 C. and make up to exactly 1,000 c.c. with distilled water. Tube in approximately 10 c.c. portions in clean, sterile test tubes. Sterilize by the intermittent method.

Materials.—If possible, select a sample of Witte peptone giving solutions of least color. Use only the purest dextrose. It is absolutely essential that the potassium phosphate should be chemically pure K_2HPO_4 . Several samples that we have received, which were labeled K_2HPO_4 by reliable dealers, have been found to be KH_2PO_4 , or mixtures of mono and dibasic phosphates. The test tubes used should be either old but unetched tubes of ordinary glass or, preferably, those made of Jena glass and steamed before sterilization.

Incubation Temperature.—30 C.

Incubation Period.—At least three days; five days is recommended.

Paranitrophenol Solution.—Dissolve 0.2 gm. re-crystallized paranitrophenol in 30 c.c. alcohol and dilute to 500 c.c. with distilled water.

Methyl Red Solution.—Dissolve 0.1 gm. methyl red in 300 c.c. alcohol and dilute to 500 c.c. with distilled water.

Procedure in Testing Cultures.—Pour about 5 c.c. of the 10 c.c. of culture into a clean, clear glass test tube and add 2-4 drops of paranitrophenol to one portion and 1-2 drops methyl red to the other. Record the color change. If the color change in any case is doubtful, compare the tint with those of a set of tubes containing solutions of known hydrogen ion concentration. For the preparation of such solutions, see the monograph by Michaelis.⁸

We have not found the use of such standards necessary, but we recommend their use if the range of color changes of the indicators is not familiar.

With the method here outlined, we tested all cultures available, of which we had 20 low ratio, 159 high ratio, 8 ∞ ratio, and three "atypical" strains. In the first test paranitrophenol was used. All the "low ratio cultures" were distinctly acid. All the "high ratio cultures" were distinctly alkaline. All the " ∞ ratio cultures" were distinctly alkaline. Of the "atypical" cultures 179-22og and 452-22oh were acid, while 454-22of was alkaline.

The six Kligler cultures were again tested and agreed as before, except that 451-22ol, which is a distinct high ratio organism, failed to react distinctly alkaline once in three trials. In this case, its hydrogen concentration was 7.8×10^{-8} , a reaction easily distinguished with methyl red from the brilliant color given by a low ratio culture.

In order to check these results, the series of 190 cultures were again tested. This time the tests were made by one of us who knew the individual history of only two of the cultures and who consequently was not prejudiced in his judgment. The incubation period was also shortened to three days, and the applicability of methyl red was tested. Without exception, the "low ratio cultures" were perfectly colorless to paranitrophenol and brilliantly red to methyl red. Without exception, the "high ratio cultures" were distinctly colored with paranitrophenol. All but five were distinctly yellow to methyl red. These five were in the neutral tints, but so far below the distinct and brilliant red given by the "low ratio cultures" that there was no difficulty whatever in distinguishing them. Of the " ∞ ratio cultures," two were alkaline and six acid to both indicators. Of the atypical cultures 454-22of was alkaline, and 452-22oh and 179-22og were acid to both indicators.

The " ∞ ratio cultures," it will be noticed, reacted acid to paranitrophenol in the preliminary test under anaerobic conditions. In

8. Das Wasserstoffionen-Konzentration, 1914.

the second aerobic preliminary test, part were alkaline, part acid. Reducing the sugar content and allowing five days' growth in the first "final" test brought them all to an alkaline reaction. In the last experiment, three days' growth left some alkaline and others acid. We have a plausible explanation of this inconsistency, not based entirely upon the results shown in Chart 2, but well illustrated by those curves. Inspection of these curves shows that the " ∞ ratio culture" rose to a higher point than either of the "high ratio cultures." Unlike the "low ratio cultures," however, it found 0.5 percent dextrose in this particular medium insufficient for the attainment of a limiting acidity. Consequently, a reversion took place. Had the oxygen supply been shut off, as it was in the first experiment, the reversion would not have taken place. But even under aerobic conditions, as in the second experiment, 1 percent of sugar allowed the production of so much acid that even seven days did not suffice to revert the hydrogen ion concentration to a point alkaline to paranitrophenol. Reducing the sugar content and allowing five days' activity brought all the cultures to a point alkaline to paranitrophenol, but the three days' growth in the last experiment was not sufficient for an extensive reversion.

Were we to study the conduct of these " ∞ ratio organisms" in more detail, it is highly probable that a very simple method could be devised for distinguishing them from the "high" and "low ratio cultures." This, however, is hardly necessary at present, since other cultural characteristics, such as their peculiar growth on agar, are sufficient to prevent their being mistaken for true members of the colon-aerogenes family.

In regard to the three "atypical" cultures, we shall draw no conclusions at present.

On the other hand, the results with the "high" and "low ratio cultures" leave no doubt that a set of very simple conditions has been established which permits a differentiation of these two groups by a very simple test. In all the cases tested under anaerobic conditions, the correlation between gas ratio and indicator test was perfect. In the medium modified to suit simpler cultural methods under aerobic conditions, the correlation was again perfect.

At present we know of no single test which correlates with the gas ratio to this extent. We have "high" and "low ratio cultures" which produce indol, liquefy gelatin, produce similar pigments, and

ferment the same carbohydrates and their derivatives; but a close study of the gas-producing powers of these two classes reveals striking differences. Accurate estimations of their acid-producing and acid-resisting powers reveal striking differences. We believe that in these differences in the gas ratio, on the one hand, and in the acid production, on the other, we have indices of fundamental differences in metabolism.

With these differences established, it only remained to find the conditions and the means for making them evident to the eye. Then a simple diagnostic test resulted. Its value lies in the established correlation between the physiologic characteristics it detects, and the sources of the organisms possessing these characteristics. Its reliability rests, not only upon the established correlation with the gas ratio, but upon the fact that it is based upon somewhat firmer foundations than the chance observation of some minor physiologic peculiarities.

But with all this in its favor, a final word of caution is necessary. The three "atypical" strains studied, we have not yet classified by either gas ratio or indicator test. It is highly probable that very old cultures, such as these were, or strains found in nature will have "atypical" properties and will frequently occur in extensive collections. Judgment of these requires the greatest caution, and the application to such cultures of any biochemical test, such as the one we have outlined, unless it be applied with sufficient care to give it the dignity of the most accurate quantitative measurement available, can hardly be considered worthy of serious consideration.

Having emphasized this caution, we feel justified in urging that the method here developed be subjected to careful investigation by others and in connection with accurate gas determinations. If it differentiates the cultures in other collections of the colon-aerogenes group as well as it has ours, it should prove a useful method.

Since one of the requisites of a routine method seems to be rapidity, it may be well to note that exclusive of the time required for the preparation and inoculation of the tubes, fifty tests can be made in an hour. The incubation period may seem long to those impatient for results, but we urge a five-day period at 30 C. with no apology.

Many applications of the general principles involved in this method have already suggested themselves, and work upon some of these is now in progress.

We seize this opportunity to express our appreciation of Mr. Rogers' help in caring for the cultures and in making the large number of inoculations required.

SUMMARY

The diagnostic value of the gas ratio and the sanitary significance of the correlation between gas ratios and the sources of the organisms of the colon-aerogenes family studied in this laboratory have suggested the utility of a simple test which will distinguish the two main groups.

By the use of the hydrogen electrode, the changes in hydrogen ion concentration have been followed in various cultures of the bacteria in question. By such studies differences in the metabolism of different species have been detected, and the correlation of these differences with the gas ratios has been established.

Simple conditions have been found under which metabolism can be so controlled that the hydrogen ion concentrations of cultures of one group can be made to diverge widely from those of the other group. These differences can be sharply distinguished by means of paranitrophenol or methyl red. In this way a simple diagnostic test has been established the results of which correlate perfectly with the gas ratios of the two main groups of the colon-aerogenes bacteria.

CHROMOGENESIS IN CULTURES OF SPOROTRICHA *

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(WITH PLATES 2 AND 3)

The pigmentation of fungi has long attracted attention for various reasons. Many are highly colored and therefore readily attract the eye and excite the interest of the observer. To the systematist, color has furnished an easy means of classification, and biologic nomenclature is laden with color words. It is generally admitted however that a color basis is very unsatisfactory for purposes of classifying or naming organisms. As Buller¹ states, it would be interesting if some law of progressive coloration could be discovered; but no attempt to work out the phylogensis of the color of spores has yet been made. Buller thinks that colorlessness is the primitive condition of spores and that pigments are only gradually developed, probably by a series of mutations.

As to the significance of color in fungi, little can be said. Pigments in certain plants may be protective, especially against light; but certainly many pigments are not thus protective, for they occur in places never exposed to light. Such pigments probably have no significance or purpose, and represent merely normal products of metabolic processes.

Since at present the subject of pigmentation of lower organisms is not well known, especially from the chemical standpoint, and further work along this line is needed, I wish to record some data on pigmentation which have been accumulating for several years in connection with certain studies that I have been making on a number of strains of the pathogenic fungus sporothrix.

In a paper² published in 1898, the pigmentation of a sporotrichum was noted by Schenck, who was the first to describe this organism and call attention to its pathogenicity for man and animals. In describing the growth on agar, he states: "In cultures of ten days and older, the growth is very thick; the surface is rough, corrugated, and stained a dark brown color, the shade at the periphery being deeper than in the center. The medium also is colored." Again,

* Received for publication May 6, 1915.

1. Researches on Fungi, 1909, p. 13.

2. Bull. Johns Hopkins Hosp., 1898, 9, p. 286.

in discussing the growth on sugar media, he states: "In cultures older than fourteen days, however, the growth continues longer and becomes heavier on glucose than on plain agar, and in all the sugar media there is more discoloration both of the growth and of the substratum." On potato, he notes that the edges of the growth become "discolored and the potato darkened." Dr. Erwin F. Smith, of the U. S. Department of Agriculture, Washington, who discussed the classification of the organism in Schenck's paper, also refers to the color, especially with respect to its significance in the differentiation of fungi.

Hektoen and Perkins,³ who reported the second case of sporotrichosis on record, refer to the pigmentation of cultures as follows: "About the seventh day the growth, which has increased somewhat in thickness, becomes light-brownish in color, the margins being smooth and wavy, marked by shallow transverse grooves. Still later the growth becomes distinctly, and even dark brown, the surface wrinkled and velvety, in some cases covered by a dark fuzz. The medium becomes slightly brownish"

Later reports, especially by the French workers De Beurmann and Gougerot, have noted pigmentation of the sporothrix organisms found in that country. In Gougerot's excellent article,⁴ he states that the colonies are at first whitish, with later a gradual discoloration appearing: "Milk-coffee like, chocolate-brown, or black-brown." He further states that the pigment is constant but varies in rapidity of formation.

Page, Frothingham, and Paige,⁵ in this country, who were first to identify the disease in horses, noted that the strains of sporothrix obtained from this animal likewise produced abundant dark brown or black pigment. Excellent photographs illustrating this point accompany their article.

The strains obtained from rats by Lutz and Splendore in Brazil, those from mules observed by Carougeau in Madagascar, and that obtained from a dog in Paris by Gougerot and Caraven—all were pigment producers.

After a careful search in the reports on this disease, the statement may be made that all the strains of this organism thus far isolated whether from man or animals, when grown under proper conditions on suitable artificial media, produce a more or less distinct brown or blackish pigment.

Beyond noting its mere presence, investigators have done little or no work thus far on the nature of the pigment or on the conditions under which it is formed. It was thought wise therefore to inquire somewhat minutely into these questions.

The observations were made on eight different strains, obtained originally from both human and animal lesions. Sporotricha from France were kindly given me by Sabouraud and by Gougerot of Paris. From Dr. Kren of Vienna, I obtained a strain isolated from a human case that developed in Austria. K. F. Meyer kindly gave me an organism isolated from a horse suffering from the infection. The other strains were isolated from human cases of sporotrichosis in the United States.

3. Jour. of Exper. Med., 1900, 5, p. 77.

4. Handb. d. path. Microorganismen, 1913, 5, p. 211.

5. Jour. of Med. Research, 1910, 23, p. 137.

To the naked eye, the appearance of sporothrix cultures is at times striking. Under suitable conditions, which will be more fully discussed later, the growth of the organism assumes a brown or brownish-black color. As a rule, the upper part of the culture is more intensely colored than the lower; often, indeed, the lower part of the growth just above the water of condensation is quite white while the upper part is quite black. Again different parts of the surface growth may vary markedly in color, certain areas, or colonies, being gray or white, surrounded by a black field, or the reverse may be true. Other curious and irregular distributions of pigmented and non-pigmented growths in the same culture tube may occur. They are inconstant in many respects, even under apparently identical conditions, and therefore difficult to explain.

Under apparently the same conditions, different strains do not behave alike, there being considerable variation in the time of the appearance of the pigment, its intensity, shade, and distribution. The pigment may first appear along the margin of the growth on slant media, as a blackish-brown line, and as a rule the coloration is deeper along the margins than toward the center. But there are frequent exceptions to this. The pigment may first be seen along a ridge of growth or at the crown of a slight elevation, or hillock, of growth, both of which so often occur on the slants. Later, the color may spread and involve the entire surface, or it may become permanently limited to certain regions.

The growth on all media at first is white. The time of appearance of the pigment varies greatly. In some strains it begins to be visible in about a week or ten days; in others it may not appear for three or more weeks. At times it forms rapidly, the growth in one night becoming decidedly darkened; or the pigmentation may be a very gradual process, the media becoming slowly darker over a period of weeks.

If cultures of sporothrix are examined under the microscope, it readily may be seen that the spores are the chief seat of pigment formation. In unstained preparations placed under a cover glass, the pigmented spores can be differentiated from the non-pigmented ones. In cultures showing no pigment grossly all the spores are quite colorless. In the pigmented cultures, both non-pigmented and pigmented spores are always seen. The spores have a brownish tint, which varies considerably in intensity. Tho the growth in the tube may appear black to the naked eye, the spores under the microscope

are never more than moderately brown in color. Some spores apparently never acquire any color. Some of these are probably young, for young cultures, even tho profuse, are always colorless.

Figure 1 is intended to present the color of spores, as they appear under the microscope, from a culture which was very dark brown, or nearly black, to the naked eye. To obtain the proper effect the microscopic illumination should be carefully regulated by diminishing the intensity of the light to the proper degree. It will be observed that the pigment is absolutely homogeneous, there being no suggestion of granular structure even with the highest magnification. It is also uniformly distributed in the spore. I have not observed more intensive coloring of one side, or part, of a spore than another. The mycelium when seen in mass under the microscope may appear light brown, undoubtedly as the result of pigment; but the pigment is difficult to observe under high power and in individual filaments. In the filaments are seen at times small granular masses, highly refractile, which with proper illumination appear faintly brown in color. These also probably contribute to the coloring of the growth. At times one may obtain cultures, or parts of cultures, made up of masses of mycelia and quite free from spores. Such masses to the naked eye are usually whitish or very pale yellowish-brown, and never, so far as I have observed, deep-brown or black. The latter are always rich in spores. The evidence, then, points to the presence of a small amount of pigment in the mycelial growth, but to by far the greater portion in the spores. It may be pointed out further that pigment is never seen outside the spores or the mycelial filaments in either granular or diffuse form.

In addition to the brownish-black pigment, there is also another pigment, ordinarily less conspicuous, which is yellow or yellow-brown in color. This seems to be distinct from the brown-black pigment, but the two often exist together. The yellowish pigment is commonly seen distinctly concentrated in colonies of the organism. At times, indeed, colonies growing on the surface may be conspicuously yellow, especially when viewed from below. On agar cultures, especially sugar agar, the media as a rule come to have in the course of one to three weeks the color of honey. A layer near the surface at first becomes more intensely colored, tho the coloration is never very deep; later, usually the media assume this yellow-brown, or honey-colored appearance, throughout. This pigment is evidently diffusible in the media.

It is to be noted that this coloring of the media is different from the slight alteration due to the growth of the fungus into the media. The latter may occur to a considerable degree under certain conditions and may alter somewhat the color of the involved medium.

The discoloration of the media noted by some writers is, I take it, due to the diffusion of this yellowish or yellow-brown pigment into it, and not to the blackish or black-brown pigment of the spores, which, as we shall presently see, is quite indiffusible.

An attempt was made to determine some of the properties of the pigment. For this purpose, deeply pigmented growths several weeks old were used. The mass removed from the surface of glucose agar plates was black to the eye, and portions quite free from the media were subjected to various fluids in order to determine the solubility of the pigment. It was found to be highly insoluble. Water, alcohol 95 percent, and absolute, cold or hot, ether, xylol, chloroform, acid alcohol, weak alkalies, and weak acids appeared to have no effect. Strong HNO_3 and H_2SO_4 quickly dissolved the entire organism and destroyed the pigment. Therefore it appears that the pigment is insoluble in aqueous solutions, and indiffusible in fat solvents, weak acids, and weak alkalies.

The pigmented spores in their staining reactions appear to behave exactly as do the non-pigmented ones. The pigment does not give the iron reaction.

The character of the media with respect to chromogenesis is important. On fluid media, if the growth is permitted to remain for some time on the surface, a thick mat forms in which a brownish or blackish pigment may appear. Ordinarily, however, the growth in the fluid sinks to the bottom in floccular form and when in this position remains uncolored. This is probably due, as will be shown later, to the diminished supply of oxygen beneath the surface of the fluid. On account of the peculiar way in which the growth occurs in fluid media, the latter are not satisfactory for the study of pigment production.

Solid media furnish more suitable conditions; for here, at least on certain kinds of solid media, pigment may be observed constantly. On ordinary plain agar, as it is usually made with meat or meat extract, some pigment is often produced, but as a rule not in abundance. On sugar media, pigment is more abundant. The statement has been made that if impure sugars are used—for example, the common, dark-colored, impure glucose—the pigment is more intense and appears earlier in the cultures. I am not sure that this is so. The growth is as a rule more abundant on such media, and this profuse growth may be the cause of the more abundant pigment.

On account of the complexity of ordinary media, it was difficult or impossible satisfactorily to analyze results obtained thereon; consequently, the growth of the sporotricha was tested on a series of synthetic media made up in a variety of ways. On media containing 2 percent pure agar plus 0.5 sodium chlorid, there is always some growth but it is very slight. However, even here, slight but distinct brown or black pigment may be produced at times. The growth appears after a few days as a white layer on the surface but soon stops altogether. There is never formed the thick, heavy, corrugated growth seen on ordinary plain or sugar agar. On media containing agar 1.5 percent, sodium chlorid 0.5 percent, and pure glucose 2.0 percent, the growth is also very scant, being little or no more than in the absence of glucose. The pigment formation is also slight, but at times it is distinct. The same holds true for this media

if impure glucose is substituted for the pure glucose. In the following synthetic medium, agar 1.5 percent, asparagin 2 percent, MgSO_4 1 percent, K_2HPO_4 1 percent, sporotricha grow scantily, but on the whole perhaps slightly better than in the simpler media. Pigment may occasionally be formed in small amounts. If glucose pure or impure is added, the growth may be slightly accelerated, but the difference is not great; indeed, often inappreciable. Likewise, pigment production is not appreciably increased, tho it is often present in small but definite amounts. It is when the proteid constituents are added that the difference becomes more marked, the growth being then much more profuse and the pigment production more intense. After many observations, I have not been able to convince myself that more or intenser pigment is formed in the presence of impure glucose than of pure glucose.

Maltose is a most excellent medium for growth and for pigment production. Saccharose, lactose, inulin, and raffinose each furnish a fairly good medium for its growth, but apparently not as favorable either for growth or for pigment production as maltose and glucose. Media containing blood give good growth but do not to any extent alter or modify pigment production.

Sterile sliced carrot is a most excellent medium both for growth and pigment production. According to my experience, it gives more intense and more constant pigmentation than any other medium that I have tried. The color of the growth of many strains is a shiny, deep black. The carrot tissue is not stained brown or black, even by the most intense pigment producers.

Often on potato, pigment is produced in abundance, and the potato beneath the growth may become dark in color. This is not due however to a diffusion of pigment.

On sterile animal tissues, growth is abundant and usually appears as a gray covering without definite or intense pigmentation. The organism grows into the tissue slowly, not in the form of filaments, but in the form of spindles, which are found there in large numbers. These are never pigmented, tho the spores forming on the surface may show some color under the microscope.

All strains of sporotricha thus far reported have been aerobes. I have tested twelve different organisms, including strains from man and horse, and none gave any appreciable growth in anaerobic tubes. The spores remain alive for a considerable time under these conditions, as can be shown by admitting air into the tubes later. In this manner, it was shown that growth occurred after an anaerobic exposure of six weeks. At the end of three months no growth appeared. If a small amount of oxygen only is admitted to a culture tube, growth will occur but it is retarded. While growth was being tested under these conditions, it was noted that even with the best pigment-producing strains no pigment was formed, but instead the media were covered as a rule with a diffuse, pure white, often snowy growth, usually without corrugations or ridges. This was true regardless of the kind of media used. After a number of methods had been tried, it was found that simply plugging a tube immediately after inoculation with a solid rubber cork diminished the amount of available oxygen sufficiently. As a rule, in such preparations, no pigment whatever appears. Occa-

sionally a small amount may be seen underneath the diffuse white growth, but it does not appear on the surface. This is rare, however, and when present it is probably due to a slight excess of oxygen. When air is admitted, pigment will begin to form in the course of several days.

In stab cultures, no pigment ever appears in the growth in the depths of the media, while on the surface pigment may be abundant. The spores which form beneath the surface in such cultures, when examined for pigment with the microscope, are likewise always found to be colorless. This absence of pigment formation in the depths of the medium is probably due to an insufficient supply of oxygen at that place.

Experiments were designed to test the effect of light on the growth and pigmentation of cultures of sporothrix. Tubes inoculated and placed at once in an absolutely dark chamber continued to grow and to produce pigment in the ordinary manner. No essential differences were noted between the tubes kept in the dark and the control tubes placed in diffuse light. In sun light, too, no appreciable alterations were noted, tho the growth in certain tubes was somewhat retarded. The pigment soon appeared, and in intensity closely corresponded with the control tubes placed in diffuse light and total darkness.

In certain strains of sporotricha, there has been noted a striking distribution of pigment. The pigment occurs in well-defined regions, the remainder of the growth remaining white indefinitely, thus being produced what I have come to call spotted cultures. If the inoculation is scant so that individual colonies appear on the slant or plate, certain colonies are deeply pigmented and others are white. I do not know what causes this phenomenon. I have not noted it in connection with all strains; it was especially noticeable in a strain which I isolated from a human case reported by Dr. Hyde and myself in 1910. At times, in other cultures, the pigmented portions are not so clear-cut and well-defined, the white and the pigmented areas fusing more gradually.

If one carefully makes subcultures on maltose agar or carrot from the white and from the pigmented regions, or colonies, one often obtains white and pigmented cultures, respectively, which reproduce true (Fig. 2). Mixed cultures not infrequently result, but by carefully selecting the material one will obtain what appears to be a pure white and a pure black, or pigmented, strain. I have several such strains and have carried them through eleven generations, each generation growing

for at least six weeks before being again transferred. It is necessary to observe the cultures about this length of time in order to know whether or not pigment will be produced by the culture. Thus far they have bred true, the white producing white and the black producing black. Also many ordinary subcultures, made on media suitable for pigment production, likewise have thus far bred true. It is impossible of course to say what such cultures may do in the future and they should be kept under observations for years to determine this. At the present time I can state only that I have white strains which under the most favorable conditions for pigment production that we know, have failed to produce it during a period of observations of about sixteen months. The black strains, coming from exactly the same original human strain as the white, produce intensely black pigment. I have obtained from some, but not all, the black cultures some white ones. This has probably been due to a mixture of white and black in the pigmented colonies, which might easily occur.

The white and black strains differ in certain respects other than pigment production. The surface of the black cultures is more wrinkled and corrugated, whereas the surface of the white is more velvety, or appears powdered. Small, fine spicules are often present on both pigmented and non-pigmented strains. Spores are less abundant on the white than on the black cultures. The spores of the white cultures, when examined under the microscope, are always seen to be entirely free from color. The mycelia of the two are, however, not different.

When inoculated into animals (white rats), the black and the white strains appear to be equally pathogenic. Cultures from the lesions thus produced in these animals give rise to pure white and black varieties, identical with those injected.

Dr. Moon, by means of the Barber technic, isolated for me single spores from the black and from the white varieties. From these there developed white and black subcultures, respectively. Such strains have been carried through ten generations and have bred true.

As to the significance of this phenomenon, I think we should be conservative. It seems to indicate that at least certain strains of these organisms are readily subject to variations; possibly, we might apply the term mutation to such changes. I think however that we should be cautious about multiplying new varieties of organisms of this type based on slight variations in cultural or morphologic properties. On the other hand, it should be pointed out that if these white strains

continue to breed true and retain permanently their present properties, they will be different from all the strains that have been cultivated directly from the lesions in man or in animals. As has been pointed out, all these, under proper conditions, are pigment producers.

SUMMARY

All cultures of pathogenic sporotricha that have been isolated and carefully studied are pigmented when grown on suitable media.

The color varies, being black or shiny black, dark brown, chocolate brown, slight brown.

The time of the appearance of pigment on cultures varies considerably; it is usually from one to several weeks after inoculation.

Media most suitable for pigmentation are carrot (sliced and sterilized), potato, 3 percent maltose agar, and 3 percent glucose agar. Other sugars are not so useful for this purpose.

Cultures remain white in an insufficient supply of oxygen. Abundant oxygen seems to be necessary for chromogenesis.

Sunlight, diffuse light, and absolute darkness have no appreciable effect on chromogenesis.

The pigment is insoluble in water, acids, alkalies, and in fat solvents (ether, alcohol, chloroform, benzol, xylol).

The slight darkening of the media under the growth of sporothrix is probably due to a second pigment of a yellowish color produced by this organism.

The color lies almost entirely in the spores on the surface of cultures. Mycelium and spores in the depths of the medium are colorless, the latter probably because of lack of oxygen.

In certain cultures, pigmented and white regions, or colonies, appear. From such growths pure pigmented and pure white strains have been obtained.

When passed through animals (rats), these white and black strains remain pure.

Single spores from the white and the black cultures give rise to pure white and black subcultures, respectively.

EXPLANATION OF PLATES 2 AND 3

Fig. 1.—Pigmented and colorless spores from a culture of sporothrix which was quite black to the naked eye.

Fig. 2.—A. Culture of sporothrix from man which shows well defined regions of pigmented and non-pigmented growth.

B. Subculture on maltose agar made from pigmented area of culture A.

C. Subculture on maltose agar made from white areas of culture A. B and C were grown under identical conditions.

PLATE 2

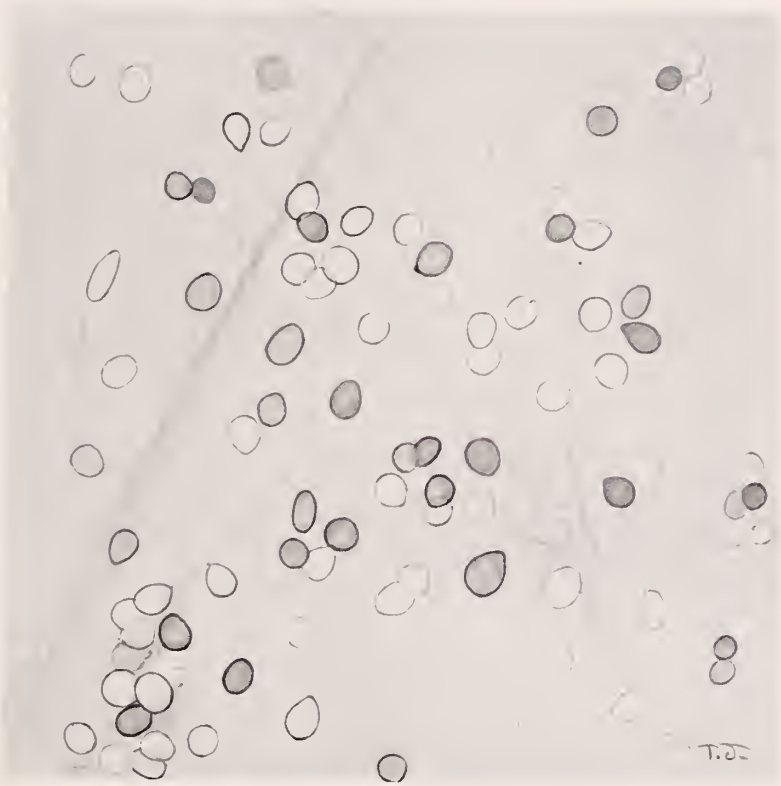


Figure 1

PLATE 3



A



B



C

Figure 2

THE NUMERICAL INTERPRETATION OF FERMENTATION-TUBE RESULTS *

M. H. MCCRADY

(From the Laboratories of the Board of Health of the Province of Quebec)

The employment of the fermentation tube in the quantitative determination of certain bacteria, particularly that of *Bacillus coli* and allied organisms in the sanitary analysis of water, milk, and other foods, has become so general, and the results of the fermentation test have acquired such significance, that much time and effort have been expended in the attempt to increase the precision of the method. Many problems relating to media, apparatus, and technic have been solved, and the fermentation test has become firmly established.

Little attention, however, has been given to the numerical interpretation of fermentation-tube results, altho an estimate of the number of fermenting organisms in the sample is the logical end of the examination. It may be of interest to know, for instance, that of 5 tubes, each inoculated with 0.1 c.c. of the sample, 4 show presence of the organism tested for; but much more important is the knowledge, afforded by this data, that the number of organisms in the sample is most probably about 1600 per 100 c.c. (instead of 800, as might have been inferred).

Closely associated with this question of number, is the question of precision. Comparison of results, whether one with another, or with a Standard, always involves this question of precision. For instance, suppose with one medium, 85 out of 100 tubes, each inoculated with 1 c.c. of the sample, show presence of the organism; while with another medium, only 75 out of 100 tubes are "positive." Can the difference between these two results be considered really significant?

Other questions are involved in this problem of numerical interpretation, but those of number and precision are by far the most important, and demand the first consideration.

Professor Phelps¹ has approached one phase (that of averages) of the problem, but the general problem of numerical interpretation has

* Received for publication May 8, 1915.

1. Am. Pub. Health Assn. Rep., 33, p. 9.

not, to the writer's knowledge, been discussed; and the considerable variation which obtains in laboratory practice in the use of the fermentation test, and in methods of expressing results, indicates a degree of uncertainty regarding the real significance of the results.

The present study is an attempt to define the factors which enter into this problem, and to indicate some of the more important applications of the mathematical analysis to laboratory practice.

For convenience of discussion, the problem as applied to water-analysis will be considered; and *Bacillus coli*, capable of fermenting the medium employed, will be the fermenting organism.

The ordinary method of expressing individual results will be followed; that is, as a fraction, the denominator denoting the number of trials, and the numerator the number of these trials which gave positive results. Thus, "2/5 in 1 c.c." means that two of five tubes, each inoculated with 1 c.c. of the sample, gave evidence of the presence of *Bacillus coli*.

The quantity of sample is assumed to be 100 c.c., but, as will be shown later, considerable variation from this quantity will not appreciably affect the results obtained on this assumption.

GENERAL THEORY

CASE 1—ONE DILUTION, ONE TUBE

The Result is Negative.—Suppose one single bacillus coli is contained in the sample of 100 c.c., and suppose one fermentation tube is inoculated with 1 c.c. of the sample.

At the moment of withdrawing this 1 c.c. of the sample, the single organism may be in this 1 c.c. or it may be in any other of the remaining 99 c.c. of the sample. There is no reason to believe that any one volume rather than any other volume will be favored with the presence of the organism. Consequently, the chances are 99 out of 100 that the organism will not be in the particular 1 c.c. withdrawn. Or, in the language of Probability, in which certainty is expressed by unity, the probability that the organism will not be contained in the 1 c.c. withdrawn is equal to 99/100, or .99. Or, to use still another mode of expression, if a great number of samples, each containing one bacillus coli, were examined in this manner, about 99 percent of the results would be negative, and the greater the number of such samples examined, the nearer would the percentage of negative results approach this figure.

Now, to illustrate the next step, suppose two coins are tossed. Of course, each coin will turn up head about half the time in the long run, and the probability of this event is then said to be $\frac{1}{2}$ or 0.50 for each coin. But, according to the well-known principle of compounding separate probabilities (a principle demonstrated in any text-book of algebra), the probability of both coins turning up heads at the same throw is equal to the product of the separate probabilities, or $(\frac{1}{2}) (\frac{1}{2}) = \frac{1}{4} = 0.25$. That is, in the long run, double heads will appear about once out of every four throws.

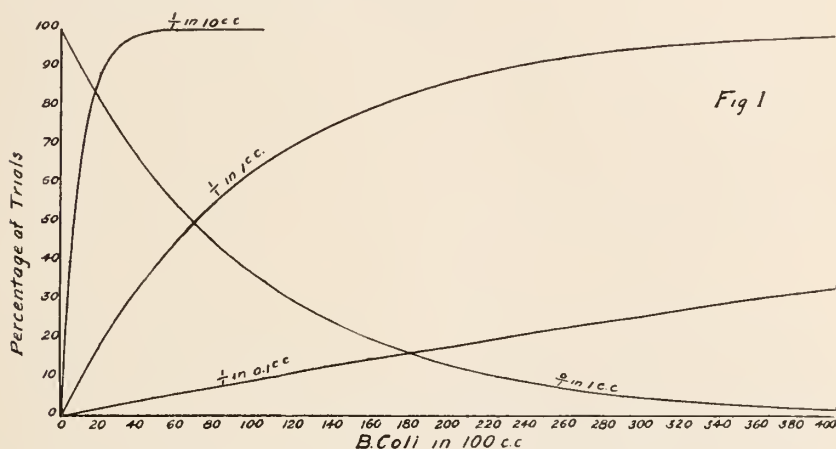


Fig. 1.—Showing the percentage of trials which will give the results indicated on the curves when certain numbers of *B. coli* are contained in the sample of 100 c.c.

Now suppose two *B. coli* are in the sample. The probability of each organism's not being contained in the 1 c.c. withdrawn for the fermentation test has been shown to be (0.99). Then by the principle just illustrated, the probability of neither organism's appearing in this 1 c.c. is equal to the product of the separate probabilities, or $(.99) (.99) = .9801$. And if a great number of such samples were examined, about 98.01 percent of the results would be "0/1 in 1 c.c."

In general, if V represents the number of volumes in the sample, and x the number of *B. coli* in the sample, and one volume is withdrawn, the probability that this volume will contain no *B. coli* is given by

$$\left[\frac{V-1}{V} \right]^x$$

Thus, when 1 c.c. of the sample is withdrawn for the test, V becomes 100 and the formula becomes $\left[\frac{99}{100} \right]^x$. When a 10 c.c. quan-

tity is withdrawn, V becomes 10 (there are ten 10 c.c. volumes in the sample), and the formula becomes p^9).

By plotting values of $(.99)^x$ against given values of x , the curve "0/1 in 1 c.c.", of Fig. 1, is obtained. This curve shows, at a glance, the probability of obtaining the result "0/1 in 1 c.c.", when any given number of *B. coli* are contained in the sample of 100 c.c. Thus, when 230 *B. coli* are in the sample, the test on 1 c.c. quantities of the sample will be negative 10 percent of the time, in the long run; and the probability of obtaining a "negative" is said to be 0.10.

The Result is Positive.—Of the two possible results, "0/1 in 1 c.c.", and "1/1 in 1 c.c.", one or the other is certain to occur. Since unity represents certainty, and the probability of the one result has been found to be $(.99)^x$, the probability of the other result, "1/1 in 1 c.c.", is equal to $1 - .99^x$.

In general, the same notation being used as before, the probability of the result "1/1," is given by

$$1 - \left[\frac{V-1}{V} \right]^x$$

The curve for this case, when 1 c.c. is the volume withdrawn, is also shown in Fig. 1, (the curve "1/1 in 1 c.c."). The curve tends upward, indicating that the greater the number of *B. coli* in the sample the greater the probability of obtaining a positive result.

It is to be noticed that (for reasons to be given later) any one of the curves of Fig. 1 may be used for that corresponding to the next higher dilution, by multiplying the abscissae by ten. Thus, the probability of the result "1/1 in 1 c.c." when $x = 30$, is practically identical with that for the result "1/1 in 0.1 c.c." when $x = 300$. Consequently, the curve "1/1 in 1 c.c." may be used for any of the "1/1" results in the higher dilutions by multiplying the abscissae by ten, one hundred, etc.

CASE 2—ONE DILUTION, SEVERAL TUBES

It may be demonstrated (in any text-book of algebra) that if P is the probability of an event happening at one trial, the probability of its happening p times out of $p+q$ trials is given by

$$\frac{(p+q)!}{p! q!} (P)^p (1-P)^q$$

It follows, then, that if 2 tubes are each inoculated with 1 c.c. of the sample, the probability of obtaining the result "1/2 in 1 c.c." is given

by $1.2/1.1 (1-.99^x) (.99^x) = 2(1-.99^x) (.99^x)$ for here $P = 1-.99$; the probability of obtaining a positive result at one trial. And $p=1$, for there is to be one positive result, and $q=1$, for there are to be $p+q=2$, trials.

The curve for this result, "1/2 in 1 c.c.", is shown in Fig. 3, at the right of the sheet. It indicates, for instance, that if the sample

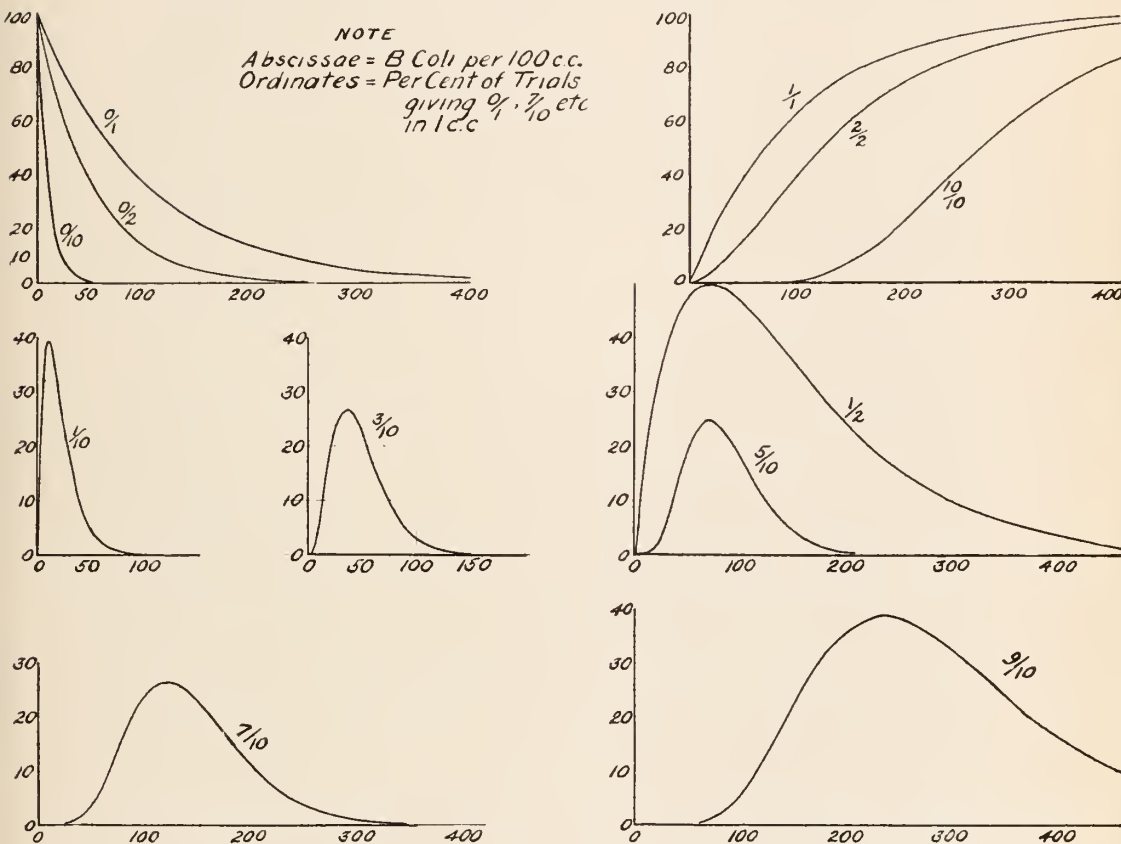


Figure 3

contains 230 B. coli, the result "1/2 in 1 c.c." will be obtained about 17.5 percent of the time, in the long run. From the other curves of Fig. 3 the result "0/2 in 1 c.c." will occur about 0.50 percent of the time and the result "2/2 in 1 c.c." about 81.0 percent of the time, respectively. Since one or the other of these results is certain to occur, the sum of these probabilities should equal unity, or 100 percent.

It is to be noticed that the number of *B. coli* which will give the result "1/2 in 1 c.c." with the greatest frequency is 69, and not 50, as might have been inferred.

Fig. 3 shows curves for many results of this case, comparison of which may prove of interest.

CASE 3—SEVERAL DILUTIONS, ONE TUBE AT EACH DILUTION

This very common case in which 10 c.c. of the sample are inoculated into one tube, 1 c.c. into another tube, 0.1, c.c. into another tube, and so on, submits very readily to calculation.

Thus, the probability of the result, "+ + —" (viz., 1/1 in 10 c.c., 1/1 in 1 c.c., 0/1 in 1 c.c.) is given by the product of the separate probabilities corresponding to the separate parts of the compound result, or

$$(1 - .9^x) (1 - .99^x) (.999^x)$$

The curve for this result, together with that for the result + — —, and for the "anomaly," (+ — +), is shown in Fig. 2.

To illustrate the use of these curves, suppose a number of samples each containing 100 *B. coli* were examined by this system of one tube at each of several dilutions, say three, 10 c.c., 1 c.c., and 0.1 c.c. From the curves it is seen that the results obtained would be about as follows: (+ — —) 32.5 percent of the time; (+ + —) 58.5 percent of the time; and the anomaly (+ — +) 3.5 percent of the time. The remaining 5.5 percent of the results would be distributed among the other possible results: (+ + +), (— + +), and (— — —).

It is to be noticed (for reasons to be mentioned later) that any one of these curves may be used for that of the next higher combination, by multiplying the abscissae by ten. Thus, the ordinate for $x=20$, in the + — — curve is practically identical with that for $x=200$, in the (+ + —) curve. (For large values of x the curve (+ — —), with abscissae multiplied by ten would correspond more nearly to the curve (+ + — —), with formula $(1 - .9^x) (1 - .99^x) (.999^x) (.9999^x)$, but for smaller values of x , the last factor, $(.9999^x)$ is practically equal to unity and may be disregarded.)

The highest ordinates of the curves for this case correspond to the numbers of *B. coli* 23, 230, 2300, etc., and in the case of the anomalies, to 9, 90, etc., approximately.

CASE 4—SEVERAL DILUTIONS, SEVERAL TUBES AT EACH DILUTION

This case, altho more complicated, may be readily analyzed. The formulae are made up by simply compounding the separate probabilities of the separate parts of the compound result, just as was done in Case 3.

Thus, the probability of obtaining the result "2/2 in 10 c.c., 3/10 in 1 c.c., 0/10 in 0.1 c.c." is given by:

$$\left[(1 - .9)^2 \right] \left[120 (1 - .99)^3 (.99)^7 \right] \left[(.999)^{10} \right]$$

Such formulae are very easily reduced with the aid of a table of logarithms; and, by plotting a few of the ordinates, the general shape of the curve for any result may be readily determined.

This Case 4, of course, includes the cases already discussed.

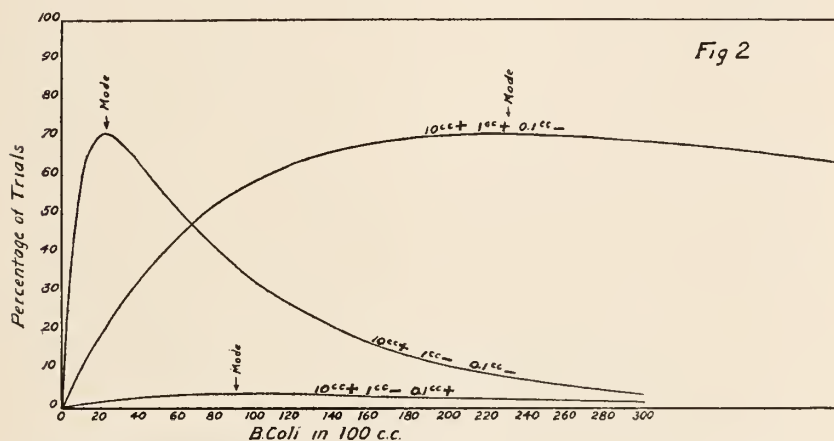


Fig. 2.—Showing the percentage of trials which will give the results indicated on the curves when certain numbers of *B. coli* are contained in the sample of 100 c.c.

ASSUMPTIONS INVOLVED IN THESE FORMULAE

Quantity of sample.—The formulae given assume the quantity of sample to be 100 c.c. But in the expression $\left[\frac{V-1}{V} \right]^x$, the basic factor of all the formulae, even though V varies, so long as x varies proportionately, the value of the factor remains practically unchanged. Thus, suppose one sample of 100 c.c. containing 50 *B. coli*, and another sample of 80 c.c. containing 40 *B. coli*. The factor becomes, for these two cases, $(.99)^{50} = .6050$ and $\left[\frac{79}{80} \right]^{40} = .6046$, or practically identical values.

In fact, a great variation, in excess, from 100 c.c., will have little effect on this factor. Thus, suppose one sample of 100 c.c. containing 100 *B. coli*, and another of 10,000 c.c. containing 10,000 *B. coli*. The factor becomes, for these two cases, $(.99)^{100}=.366$ and $(.9999)^{10,000}=.368$, respectively. This fact accounts for the facility with which one curve of Fig. 1, Fig. 2, or Fig. 3 may be employed for the curve of the next higher order, as has already been noted under Case 1 and Case 3.

Consequently, the variation in quantity of sample from 100 c.c. obtaining in ordinary laboratory practice will have little effect upon these formulae. To be sure, in special cases the variation may have to be allowed for, but in general the formulae may be applied directly to laboratory results.

Replacement.—When more than one volume is to be drawn from the sample, these formulae demand that for each draw the initial conditions must be the same. That is, after the first volume has been drawn, this volume, together with its contained *B. coli*, must be replaced in the sample-bottle before drawing the next volume. Such a procedure is obviously impossible in practice.

But perhaps, when the first volume has been drawn, it may be assumed that a proportionate number of the *B. coli* have also been drawn in this volume. If so, since both V and x have varied proportionately, the value of the general factor $\left[\frac{V-1}{V}\right]^x$ has remained practically unchanged.

But even if this assumption is not justified, calculation will show that the error due to this non-replacement is, in general, negligible. (Some experiments, to be described later, show quite clearly the negligible character of this error.)

For ordinary practical work, even when small numbers of *B. coli* are in the sample, these formulae may be employed. But in certain special work, and wherever extreme accuracy is desired, it may be necessary to construct a new set of formulae which will allow for non-replacement, as is done later in the discussion of the recently established United States Treasury Standard for waters supplied by common carriers.

APPLICATIONS

THE "MOST PROBABLE NUMBER"

Its Significance.—In all sampling, whether of population, of chemical substance, or of bacteria, a certain line of reasoning is employed which is not always recognized.

Consider the plate method of examining a water for bacteria. Suppose 1 c.c. of the sample is used, and suppose the plate count is found to be 25. The bacterial content of the sample is then recorded as 25 per cubic centimeter. But it is recognized that any number of bacteria in excess of 24 may have been contained in that sample. In other words, the record "25 per cubic centimeter" may be in error by almost any amount. Wherein, then, lies the justification for choosing this particular number, 25, to represent the average number of bacteria per cubic centimeter of the sample? It may be said that "common sense" justifies the choice. But is the argument so simple? in fact, in the exactly analogous case of the fermentation-tube result, "common sense" does not lead one very far.

The real justification is to be found in the following line of reasoning:

If the number of bacteria in the sample of 100 c.c. is 2500, the probability of obtaining the plate count 25 from 1 c.c. of the sample is equal to P_1 (given, as may be shown,* by the twenty-sixth term in the expansion of the binomial $\left\{ \frac{99+1}{100} \right\}^{2500}$).

If the number of bacteria is 2501, the probability is P_2 .

If the number of bacteria is 2502, the probability is P_3 , and so on.

But of all these various probabilities, it is found that P_1 , corresponding to 2500 bacteria, is the greatest.

Now "common sense" may be enlisted in the form of this principle²: "Of all those values of an unknown quantity which, before the occurrence of a certain event, were equally probable, that one is after the event the most probable which, before the event, assigned to it the greatest probability."

Therefore, since 2500 is that value of the unknown quantity which assigns the greatest probability to the occurrence of the event (the

* The probability of drawing exactly k organisms in the one volume drawn from the sample of V volumes, when the sample contains x organisms, is given by the $(k+1)$ th term in the expansion of the Binomial $\left[\frac{(V-1)+1}{V} \right]^x$.

2. Johnson: Theory of Errors and Method of Least Squares, 1905, p. 18.

plate-count 25), 2500 is the most probable number of bacteria in the sample, and should be so recorded.

Now notice the exactly parallel reasoning in the case of the fermentation-tube result. Take the result "9/10 in 1 c.c.":

If the number of *B. coli* in the sample was 229, the probability of obtaining this result is equal to P^1 (given by $10 (1-.99^{229})^9$ (.99²²⁹)).

If the number of *B. coli* was 230, the probability is P_2 .

If the number of *B. coli* was 231, the probability is P_3 , and so on.

Now of all these probabilities, the greatest is found to be that corresponding to the number 229.

Therefore, by the principle just enunciated, 229 is the most probable number of *B. coli* in the sample.

It is evident, then, that these two "most probable numbers," 2500 bacteria, and 229 *B. coli*, have exactly the same status in their respective domains. Each is obtained in exactly the same manner as is the other. The fermentation-tube result, "9/10 in 1 c.c.," means 229 *B. coli* per 100 c.c. just as the plate-count "25" means 25 bacteria per cubic centimeter.

Now either of these "most probable numbers" may be in error. In the case of the plate count, for instance, the number of bacteria in the sample may be 10,000 instead of 2500. But in the long run of samples of various waters, the application of the line of reasoning described will lead to a series of "most probable numbers" which, on the whole, will strike closer to the truth than will any other series of numbers obtained in any other way. Each "most probable number" represents the one best guess afforded by the data of the analysis, no matter whether the result takes the form of a plate count, a fermentation-tube result, or a chemical proportion.

Calculation of the Most Probable Number.—In the case of the plate count, it happens that this count may be converted directly into its corresponding most probable number of bacteria. The most probable number is, so to speak, a linear function of the plate count (the plate count being usually simply multiplied by one).

But in the case of the fermentation-tube result, the most probable number of *B. coli* is a logarithmic function of this result, and recourse must be had to calculation to obtain the most probable number.

Of course, one method of obtaining the most probable number corresponding to the result, say "5/10 in 1 c.c.," is to plot the curve for

this result and then pick out the highest ordinate and take the corresponding number of *B. coli*.

But ordinarily, especially for compound results, such a method is too laborious, and some short-cut by calculation is desirable.

It may be shown that, given the result " $\frac{p}{p+q}$ in 1 volume," the corresponding most probable number is given by the solution for x of the equation

$$1 - \left\{ \frac{V-1}{V} \right\}^x = \frac{p}{p+q}.$$

Thus, for the result "5/10 in 1 c.c.," the most probable number is given by solution of the equation $1 - .99^x = 5/10$. The equation being solved, $x = 69$. This is the most probable number of *B. coli* in the sample, per 100 c.c.

(It is to be noticed that the curve "1/1 in 1 c.c.," of Fig. 1, is a graph of this general equation with V equal to 100.)

From the form of the equation, it is evident that multiple results, such as "1/2 in 1 c.c." and "5/10 in 1 c.c.," correspond to the same most probable number of *B. coli*.

Application of the formula gives some interesting information. Thus, the result "9/10 in 1 c.c." means, not 90 *B. coli* per 100 c.c., but 229 *B. coli* per 100 c.c., altho the result "1/10 in 1 c.c.," means 10 *B. coli*, as might have been presumed.

For compound results, a more complicated formula must be employed. This equation may be built up as follows:

(1) Suppose the result is " $\frac{p}{p+q}$ in 10 c.c." The equation becomes

$$(p+q) (\log .9) = \frac{p (\log .9)}{1 - .9^x}$$

(These equations are obtained by differentiating for a maximum the equation for the probability of the result.)

(2) Suppose the result is " $\frac{p}{p+q}$ in 10 c.c., $\frac{r}{r+s}$ in 1 c.c." The equation becomes

$$(p+q) (\log .9) + (r+s) (\log .99) = \frac{p (\log .9)}{1 - .9^x} + \frac{r (\log .99)}{1 - .99^x}.$$

The manner in which corresponding terms are added to each side of the first equation to build up the second equation is very apparent. To take a concrete example, suppose the result is "1/2 in 10 c.c., 3/10 in 1 c.c., 0/10 in 0.1 c.c." The equation becomes

$$2 \log .9 + 10 \log .99 + 10 \log .999 = \frac{\log .9}{1 - .9^x} + \frac{3 \log .99}{1 - .99^x} + 0.$$

and solution of this equation for x gives $x = 17$, to the nearest unit, and the most probable number of *B. coli* in the sample is 17 per 100 c.c.

These equations for compound results must be solved by "trial and error," but the work proceeds very rapidly, and is not so laborious as it may appear.

For the particular system employed by each laboratory, it would be advisable to calculate, once for all, the most probable numbers corresponding to the results which this system may give. A rapid review of past results will indicate the range of practically possible combinations for which the most probable numbers should be calculated. For instance, in Table 1 are given the most probable numbers corresponding to most of the practically possible results which may occur from the system: Two tubes at 10 c.c., ten tubes at 1 c.c., and ten tubes at 0.1 c.c. Of course, still other combinations, not included in this table, are sure to occur sooner or later, and must be given their most probable numbers; but such other combinations will not occur often.

Every Result Must Be Given Its Interpretation.—Just as every plate-count is given its most probable interpretation, so should every fermentation-tube result be given its most probable interpretation. Just as it may seem absurd to think of the logarithm of the plate count when considering the number of bacteria in the sample, so should it seem absurd to think of the result "2/3 in 1 c.c." when considering the number of *B. coli* in the sample. This result means, so far as the analytical result can signify, 109 *B. coli* per 100 c.c. and this is the record of the analysis which must be considered.

By the methods which have been described, every result, whether simple or compound (except the single result N/N, such as "3/3 in 1 c.c.") may be interpreted. Compound results, particularly, require such interpretation, for it is often quite difficult to guess the significance of such results.

Moreover, the odd result, such as the "anomaly" in the system of one tube at each dilution, must not be generally regarded with suspicion. In fact every possible result, every possible combination, is sure to be obtained sometime. Overgrowths and other cultural difficulties may be responsible for some odd results, but, in general, the benefit of the doubt must be given the chance distribution, for it is the one cause of such results which is known to be continually operating.

Calculation will often show whether the proportion of odd results obtained on a series of samples is to be suspected. Thus, the curve of Fig. 2 shows that the anomalies will occur about 3 or 4 percent of the time, on the average; therefore, a proportion of anomalies greatly in excess of this may be regarded with suspicion.

TABLE 1
GIVING THE "MOST PROBABLE NUMBERS" OF B. COLI PER 100 C.C. OF SAMPLE CORRESPONDING TO VARIOUS
FERMENTATION-TUBE RESULTS

Using Two Dilutions						Using Three Dilutions													
10 c.c.	1 c.c.	Number	10 c.c.	1 c.c.	0.1 c.c.	Number	10 c.c.	1 c.c.	0.1 c.c.	Number	10 c.c.	1 c.c.	0.1 c.c.	Number	10 c.c.	1 c.c.	0.1 c.c.	Number	
0/2	0/10	0	0/2	0/10	0/10	0	1/2	4/10	0/10	22	2/2	4/10	4/10	94	2/2	8/10	5/10	245	
0/2	1/10	3	0/2	0/10	1/10	3	1/2	4/10	1/10	27	2/2	4/10	5/10	105	2/2	8/10	6/10	275	
0/2	2/10	7					1/2	4/10	2/10	32					2/2	8/10	7/10	310	
0/2	3/10	10	0/2	1/10	0/10	3	1/2	4/10	3/10	37	2/2	5/10	0/10	61					
0/2	4/10	14	0/2	1/10	1/10	6					2/2	5/10	1/10	72	2/2	9/10	0/10	170	
							2/2	0/10	0/10	10	2/2	5/10	2/10	86	2/2	9/10	1/10	195	
1/2	0/10	4	0/2	2/10	0/10	6	2/2	0/10	1/10	16	2/2	5/10	3/10	100	2/2	9/10	2/10	225	
1/2	1/10	8	0/2	2/10	1/10	10	2/2	0/10	2/10	23	2/2	5/10	4/10	115	2/2	9/10	3/10	260	
1/2	2/10	13					2/2	0/10	3/10	30	2/2	5/10	5/10	130	2/2	9/10	4/10	300	
1/2	3/10	17	0/2	3/10	0/10	10					2/2	5/10	6/10	145	2/2	9/10	5/10	345	
1/2	4/10	23					2/2	1/10	0/10	17					2/2	9/10	6/10	395	
1/2	5/10	28	0/2	4/10	0/10	13	2/2	1/10	1/10	23	2/2	6/10	0/10	79	2/2	9/10	7/10	455	
1/2	6/10	35					2/2	1/10	2/10	31	2/2	6/10	1/10	93	2/2	9/10	8/10	525	
			1/2	0/10	0/10	4	2/2	1/10	3/10	41	2/2	6/10	2/10	110					
2/2	0/10	11	1/2	0/10	1/10	8	2/2	1/10	4/10	49	2/2	6/10	3/10	125	2/2	10/10	0/10	240	
2/2	1/10	18									2/2	6/10	4/10	140	2/2	10/10	1/10	285	
2/2	2/10	27	1/2	1/10	0/10	8	2/2	2/10	0/10	24	2/2	6/10	5/10	155	2/2	10/10	2/10	345	
2/2	3/10	37	1/2	1/10	1/10	12	2/2	2/10	1/10	33	2/2	6/10	6/10	170	2/2	10/10	3/10	430	
2/2	4/10	52	1/2	1/10	2/10	16	2/2	2/10	2/10	42					2/2	10/10	4/10	550	
2/2	5/10	69	1/2	1/10	3/10	20	2/2	2/10	3/10	52	2/2	7/10	0/10	100	2/2	10/10	5/10	720	
2/2	6/10	91					2/2	2/10	4/10	62	2/2	7/10	1/10	120	2/2	10/10	6/10	920	
2/2	7/10	120	1/2	2/10	0/10	12					2/2	7/10	2/10	135	2/2	10/10	7/10	1,200	
2/2	8/10	160	1/2	2/10	1/10	16	2/2	3/10	0/10	34	2/2	7/10	3/10	155	2/2	10/10	8/10	1,600	
2/2	9/10	230	1/2	2/10	2/10	21	2/2	3/10	1/10	44	2/2	7/10	4/10	175	2/2	10/10	9/10	2,300	
			1/2	2/10	3/10	26	2/2	3/10	2/10	54	2/2	7/10	5/10	195					
							2/2	3/10	3/10	65	2/2	7/10	6/10	215					
			1/2	3/10	0/10	17	2/2	3/10	4/10	77	2/2	7/10	7/10	235					
			1/2	3/10	1/10	21	2/2	3/10	5/10	88									
			1/2	3/10	2/10	26					2/2	8/10	0/10	130					
			1/2	3/10	3/10	31	2/2	4/10	0/10	46	2/2	8/10	1/10	150					
							2/2	4/10	1/10	57	2/2	8/10	2/10	170					
							2/2	4/10	2/10	69	2/2	8/10	3/10	195					
							2/2	4/10	3/10	81	2/2	8/10	4/10	220					

These "most probable numbers," when less than 100, are correct to the nearest unit. When over 100, they are correct to the nearest 5 units.

But unless there is overwhelming evidence in favor of rejecting any particular result, it should be recorded, and given its most probable interpretation.

THE PRECISION OF A RESULT

Obvious Limits.—A notion of the precision of a result may be obtained simply by inspection of the curve for this result. Thus, suppose the result "5/10 in 1 c.c." Inspection of the corresponding curve

of Fig. 3 shows that the practically possible numbers of *B. coli* have a range from about 15 to about 200 per 100 c.c.

Moreover, by an extension of the reasoning, already outlined, for the determination of the significance of the "most probable number," it may be shown that the relative heights of the ordinates of the curve give the relative probabilities that the corresponding abscissae were responsible for the result. Consequently, the general shape of the curve indicates roughly the degree of confidence which may be assigned to the inclusion of x within certain limits. Thus, with the result "1/10 in 1 c.c.," it is quite certain that the number of *B. coli* lies within the range 0 to 50. But with the result "9/10 in 1 c.c.," it is far from certain that the number lies between even 200 and 300.

Again, consider the multiple results "1/2 in 1 c.c." and "5/10 in 1 c.c." These results both correspond to the same "most probable number," but the precision of the one is quite different from that of the other. And inspection of the corresponding curves gives some indication of the extent to which the respective precisions differ.

(A more elaborate and exact method of determining the precision, based on this general principle, is given further on.)

Significance of Certain Results.—Suppose a stream sampled at two points. Suppose the result on the upper sample to be "0/1 in 1 c.c." and the result on the lower sample to be "1/1 in 1 c.c." Can the difference between these two results be considered significant? Obviously, from the manner in which the two corresponding curves of Fig. 1 overlap, the presumption in favor of the greater *B. coli* content obtaining at the lower point is very slight indeed.

Suppose the stream contained uniformly 69 *B. coli* 100 c.c. The probability of obtaining the result "0/1 in 1 c.c." on the upper sample and the result "1/1 in 1 c.c." on the lower sample, is given by the product of the two separate probabilities, or

$$(.99^{69}) (1 - .99^{69}) = (.5) (.5) = .25$$

This same probability holds for obtaining the result "0/1 in 1 c.c." on the lower sample and the result "1/1 in 1 c.c." on the upper sample. The sum of these two probabilities is 0.50, and therefore, half the time, when sampling at these two points, the result on one sample will be positive and on the other sample negative. One quarter of the time both will be positive, and one quarter of the time both will be negative. (It will be noticed that this case is exactly analogous to that of tossing two coins, previously discussed.)

Again, suppose the upper stream contained 160 *B. coli* per 100 c.c., and the lower stream only 90. The probability of obtaining the result "0/1 in 1 c.c." at the upper point, and "1/1 in 1 c.c." at the lower point, is given by

$$(.99^{160}) (1 - .99^{90}) = (.2) (.6) = .12$$

Consequently, about once every eight times that these conditions obtain, the inference would be that the *B. coli* content of the lower stream was greater than that of the upper stream, and this despite the fact that the upper stream contained nearly twice the number of *B. coli* contained in the lower stream.

These examples show very clearly the necessity of an exhaustive examination of every sample (the employment of many trials), as well as of frequent sampling, especially when results are to be compared. They also indicate how, by a few simple calculations, some notion of the precision of a result may be obtained. In the instances given, such calculations have revealed beyond question the utter untrustworthiness of a system of one tube at each dilution in work of a closely comparative nature.

Precision by Bayes' Theorem.—By a principle of Probability known as Bayes' Theorem,³ it may be demonstrated that, given the result "1/2 in 1 c.c.", the probability that the sample contained less than k *B. coli* per 100 c.c. is given by dividing the sum of the ordinates from $x=0$ to $x=k-1$, of the curve for this result, by the sum of all the ordinates of the curve. This principle is an elaboration of that previously mentioned, namely, that the relative heights of the ordinates give the relative probabilities that the corresponding numbers of *B. coli* were responsible for the result obtained.

The summation of the ordinates may be effected as follows: For any result of the form " $\frac{p}{p+q}$ in 1 c.c.", the sum of all the ordinates of the curve is given by

$$\frac{(p+q)!}{p!q!} \left[\frac{1}{1-.99^q} - p \frac{1}{1-.99^{(1+q)}} + \frac{p(p-1)}{2!} \frac{1}{1-.99^{(2+q)}} \dots \dots \text{to } p+1 \text{ terms} \right]$$

And the sum of the ordinates from $x=k$ to $x=\infty$, inclusive, is given by

$$\frac{(p+q)!}{p!q!} \left[\frac{.99^{kq}}{1-.99^q} - p \frac{.99^{k(1+q)}}{1-.99^{(1+q)}} + \frac{p(p-1)}{2!} \frac{.99^{k(2+q)}}{1-.99^{(2+q)}} \dots \dots \text{to } p+1 \text{ terms} \right]$$

3. Todhunter: History of the Theory of Probability, 1865, p. 294; Poincaré: Calcul des Probabilités, 1912, p. 154.

As an example, take the result "1/2 in 1 c.c." Here $p=1$, $q=1$, and the sum of all the ordinates is

$$2 \left\{ \frac{1}{1-.99} - \frac{1}{1-.99^2} \right\} = 99.5025$$

and the sum of the ordinates from say $x=300$ to $x=\text{infinity}$, inclusive, is

$$2 \left\{ \frac{.99^{300}}{1-.99} - \frac{.99^{\infty}}{1-.99^2} \right\} = 9.566$$

The sum of the ordinates to the left of $x=300$ is then

$$99.503 - 9.566 = 89.937$$

Therefore the probability that the number of *B. coli* is less than 300 per 100 c.c., is $\frac{89.937}{99.503}$, or the odds are 89.937 to 9.566, or about nine to one, that the number of *B. coli* is less than 300 per 100 c.c. rather than 300 or more.

For the O/N curves, these formulae reduce very simply. Each ordinate becomes the probability that the number of *B. coli* in the sample is equal to, or greater than, the corresponding abscissae. Thus with the result "0/2 in 1 c.c.", its curve (Fig. 3) shows that the ordinate corresponding to $x = 150$ is about 0.05, and consequently the odds are about 95 to 5 that the number of *B. coli* in the sample is less than 150 per 100 c.c.; or, in other words, in analyzing a large number of samples of various waters, about 95 percent of those samples which give the result "0/2 in 1 c.c." will have contained less than 150 *B. coli* per 100 c.c.

There is a certain assumption involved in this principle which must be recognized. This assumption is that all numbers of *B. coli* are equally probable; that is, that in the long run of samples, one number of *B. coli* will appear about as often as any other number.

It is to be noticed that this assumption is the same, in kind, as is involved in the determination of the "most probable number." There, it is assumed that the "most probable number," so determined, is as probable as any other number. To illustrate, suppose a sample of sewage gave a plate-count of 25 per c.c. This count would immediately be regarded with suspicion, because the analyst's experience tells him that the bacterial content of 25 per c.c. is not as probable as many other bacterial contents. Consequently, he does not trust the "most probable number." But whenever the analytical result is accepted, this attendant assumption is accepted also.

But in the application of the principle under consideration, the assumption is bolder. It demands that all the various numbers of *B. coli* shall be equally probable.

(Before proceeding further, it must be noted that, altho the curves theoretically extend to infinity on the right, those ordinates which are great enough to affect the summations all lie within a fairly short range. Thus, with the curve "0/2 in 1 c.c.", the ordinates beyond $x=300$ are practically equal to zero, and may be neglected; so that, for this result, Bayes' Theorem demands only that numbers of *B. coli* from 0 to 300 shall be equally probable.)

But considerable justification may be found for this apparently bold assumption. Experience teaches that, in general, one number of *B. coli* does occur about as often as another. To be sure the filter operator will have good reason to doubt the applicability of the assumption to the result "2/3 in 1 c.c.", on the effluent of his filter, for experience tells him that the larger values of x at the right of the curve for this result are not as likely to occur as are the smaller values at the left of the curve. But, on the other hand, the State or Provincial analyst, who is examining a great number of samples of various waters, may perhaps feel justified in assuming that, roughly, over a rather wide range, one number of *B. coli* is turning up about as often as any other number.

"When the probability is unknown," says Laplace,⁴ "we may equally suppose it to have any value between zero and unit." Again, according to Edgeworth, quoted by Pearson,⁵ "The assumption that any probability constant about which we know nothing in particular is as likely to have one value as another, is grounded upon the rough but solid experience that such constants do, as a matter of fact, as often have one value as another."

In any event, despite the difficulty involved in the assumption upon which Bayes' Theorem is based, the application of the theorem to fermentation results appears to offer some interesting possibilities. It is the nearest approach that may be made to a concrete notion of the precision of a result. The "most probable number" is extremely important, but it is known to be subject to error. The shape of the curve gives some idea of the facility of this error. But calculation of the precision affords, wherever the assumption involved is even approximately justified, a degree of assurance which permits of a fairly definite

4. Introduction, *Théorie Analytique des Probabilités*, 1814.

5. *Grammar of Science*, 1911, p. 146.

conclusion regarding the *B. coli* content of the sample. To say the least, there is a certain amount of satisfaction to be derived from thus placing, so to speak, a limit on the error.

It is to be noticed that this method of calculating the precision of a result is analogous to that employed in many other branches of science, such as Physics, Astronomy, Precise Surveying, in fact, wherever the Method of Least Squares is employed.

COMPARISON OF THE PLATE METHOD AND THE FERMENTATION-TUBE METHOD OF ANALYSIS

Before the more recent fermentation-tube methods of analysis came into general use, the litmus lactose agar plate method of estimating *B. coli* was rather widely employed. And there are, perhaps, not a few workers who are still inclined to favor the plate method on the grounds that greater precision characterizes the results obtained by this method.

Suppose there were available some plate method comparable with the fermentation-tube method, in respect of faculty of growth of *B. coli*.

Let a number of samples, each containing 100 *B. coli*, be examined by both methods, as follows: (1) in each of two plates, 1 c.c. of the sample; (2) in each of ten fermentation-tubes, 1 c.c. of sample.

The percentage of times that 0 *B. coli*, 1 *B. coli*, 2 *B. coli*, etc., will appear on the two plates, is given by the first, second, third, etc., terms of the expansion of the binomial $\left[\frac{49 + 1}{50} \right]^{100}$.

The percentage of times that the results 1/10, 2/10, etc., will occur with the tubes may be calculated by the methods already given.

When 2 *B. coli* appear on the two plates, the most probable number in the sample is, of course, 100 per 100 c.c.; when 4, 200 per 100 c.c., etc.

The most probable numbers corresponding to the fermentation-tube results may be calculated by the methods already given.

In Table 2 are shown these calculations: the percentage of times that the various results will occur, in the long run, together with the most probable numbers of *B. coli* corresponding to these results.

Comparison of these figures shows clearly that the advantage in precision is with the tube method of analysis, when ten tubes are used

against two plates; for it will indicate numbers of *B. coli* which are closer to the true number, 100, oftener than will the plate method.

With a much greater number of *B. coli* in the samples, however, the system of two plates might give the better results, for then the necessity of using dilutions would decrease the precision of the tube method. But in practice, the numerous other bacteria usually associated with large numbers of *B. coli* tend to over-grow and obscure the latter, to the end that a limit is defined, beyond which quantities of sample less than 1 c.c. must be plated, with a consequent decrease in the precision of the plate method.

TABLE 2
A COMPARISON OF THE MOST PROBABLE NUMBERS OBTAINED ON THE ONE HAND BY THE PLATE METHOD AND ON THE OTHER BY THE FERMENTATION-TUBE METHOD OF ESTIMATING *B. COLI*

Plate Method			Fermentation-Tube Method		
Result (Count) (Sum of 2 Plates)	Percentage of Time	Most Probable Number <i>B. Coli</i>	Result	Percentage of Time	Most Probable Number <i>B. Coli</i>
0.....	13.26	0	0/10.....	0.00+	0
1.....	27.98	50	1/10.....	0.07	10
2.....	27.33	100	2/10.....	0.58	22
3.....	18.23	150	3/10.....	2.69	35
4.....	9.00	200	4/10.....	8.16	51
5.....	3.53	250	5/10.....	16.95	69
6.....	1.14	300	6/10.....	24.47	91
Over 6.....	0.57	Over 300	7/10.....	24.22	120
			8/10.....	15.73	160
			9/10.....	6.05	229
			10/10.....	1.05	Over 229

In any event, the tube method of analysis is evidently quite as precise as other methods in general use, especially when ten tubes are employed. (A convenient device by means of which ten tubes may be employed as a unit is described further on.)

EXPRESSION OF RESULTS

Averages.—Because of the fact that the number of *B. coli* in a sample is a logarithmic function of the frequency of their appearance in the volume drawn, the problem of averaging results is very difficult of solution. It is somewhat analogous to that of finding the average of a series of numbers when the average of their logarithms is given.

Take the two results “1/10 in 1 c.c.”, and “9/10 in 1 c.c.”. The average of the results is “5/10 in 1 c.c.”, the most probable number corresponding to which, is 69 *B. coli* per 100 c.c. But the most prob-

able number corresponding to the first result, "1/10 in 1 c.c.", is 10 B. coli, and that corresponding to the other results is about 230 B. coli. The average of these two most probable numbers is 120, a figure quite different from the figure 69.

Indeed, it is difficult to find any form of average which will answer all requirements. After considerable work on the problem, the writer is inclined to favor the average of the "most probable numbers" (just as is done with plate counts) for all results involving the use of more than one tube at each dilution. For the latter case, where only one tube at each dilution is used, this method of averaging "most probable number" appears to break down, but the method offered by Professor Phelps⁶ seems to give fairly good averages. This method assumes the frequency of the appearance of the organism in the volume tested to vary directly with the numbers of organisms in the sample, an assumption which is not in accordance with theory. Yet, so long as anomalies are excluded from the calculation, the average by this method will not often be greatly in error, except when very short series are averaged. Calculation indicates that averages (by Phelps' method) of long series of results will be rather uniformly in excess of the true average, while averages of short series may be either in excess or defect of the true average. (Further study on this problem of averaging is in progress.)

Expression of Results.—This brief discussion shows clearly the futility of expressing results by the form of average, "B. coli present in 1 c.c. 75 percent of the time." Such an expression conveys practically no information, and is quite worthless for purposes of comparison.

On the whole, since averages are so untrustworthy, it appears that the best method of expressing results is that of giving each result in full, together with the corresponding most probable number, thus:

10 c.c.	1 c.c.	0.1 c.c.	B. coli per 100 c.c.
$\frac{1}{2}$	$\frac{7}{10}$	$\frac{1}{10}$	16
+	+	—	230
—	+	—	9

Such expressions give all the data of the result, so that one may determine the precision of the result, and also express the result as a concrete number of B. coli. The advantage, to the uninitiated, in having these concrete numbers given is obvious. What analyst has not labored with officials in an attempt to explain the fermentation-tube

6. Am. Pub. Health Assn. Rep., 1907, 33, p. 9.

result, and usually with little success? They can easily comprehend the meaning of the plate-count, but the involved, compound, fermentation-tube result, expressed as such, is not so readily understood, and indeed often appears to contain contradictions.

THE U. S. TREASURY STANDARD⁷

The recently established U. S. Treasury Standard for waters supplied by common carriers, demands that not more than one of five separate 10 c.c. quantities of the sample shall afford evidence of the presence of *B. coli*. It may be of interest to discuss the probabilities for this case.

Here the *B. coli* content of the samples will be rather small, as a rule, and moreover the sample is considerably depleted during the examination (usually more than half of the sample being withdrawn for the fermentation tests). Consequently, the assumptions involved in the general formulae already given do not hold, and a new set of formulae must be constructed for this particular case.

It may be shown that, for this case, the following formulae hold:

Probability of the result " $\frac{0}{5}$ in 10 c.c." is $(.5)^x$.

Probability of the result " $\frac{1}{5}$ in 10 c.c." is $5(.6^x - .5^x)$.

Probability of the result " $\frac{2}{5}$ in 10 c.c." is $10[.7^x - 2(.6^x) + .5^x]$.

These formulae allow for non-replacement, and require only that the quantity of sample be close to 100 c.c.

Some calculations, by means of these formulae, indicating the percentage of various results which will occur when various numbers of *B. coli* are contained in the sample, are shown below:

No. of <i>B. Coli</i> In Sample x	Pct. of Time that " $\frac{0}{5}$ in 10 c.c." Will Occur	Pct. of Time that " $\frac{1}{5}$ in 10 c.c." Will Occur	Pct. of Time that " $\frac{2}{5}$ in 10 c.c." Will Occur
0	100	0	0
1	50	50	0
2	25	55	20
3	12.5	45	36
4	6.25	33.55	
5	3.13	23.26	
6	1.56	15.52	
7	0.78	10.09	
8	0.39	6.44	
9	0.19	4.05	
10	2.54	
11	1.57	

It will be noticed from these calculations that when 4 *B. coli* are present in the sample, the sample will pass the standard about 40

7. U. S. Pub. Health Rept., 1914, p. 2959.

percent of the time. And one out of about every six samples, containing 6 *B. coli*, will pass the standard (1.56 percent plus 15.52 percent). On the other hand, one out of every five samples containing only 2 *B. coli* per 100 c.c. will fail to pass the standard.

Consequently, when it is remembered that the standard signifies a most probable limit of 2 *B. coli* per 100 c.c., it is evident that the standard method of analysis renders the standard much more lenient than might, at first glance, be supposed.

(It is to be noticed that the most probable number corresponding to the result "1/5 in 10 c.c.", viz., 2, is the same as that given by the general formulae which do not allow for non-replacement. In general, the "most probable numbers" are quite the same, whether or not non-replacement is allowed for, so far as current methods, or systems, of analysis are concerned.)

METHODS FOR OBTAINING CONSIDERABLE PRECISION

In experimental work it is often necessary to know very approximately the number of fermenting organisms contained in the sample. Now, with the aid of the formulae given, the investigator may determine beforehand the details of his experiment in order that significant results may be obtained.

For instance, if two forms of apparatus are to be compared, it is evident that more than ten tubes must be used with each form; for two results such as "1/10" and "3/10" are of no significance as regards relative facility of growth with the two forms of apparatus; the curves of these results overlap so considerably that one would almost be justified in expecting just the opposite order of inference at the next experiment.

So also when even 100 tubes are used, the plotting of a few ordinates of the curves for the results will reveal the significance of these results and indicate whether or not the differences among these results may be called "significant."

If considerable precision is desired in the result to be obtained from examination of a water supply, the use of many tubes inoculated from one large sample is preferable to the use of a few tubes inoculated from each of several small samples. For, as has been shown, if the *B. coli* contents of the small samples vary, the average of the results obtained from these small samples is not trustworthy. But

the result from the single large sample (composite, if necessary) is trustworthy according to the number of tubes employed in its analysis.

Moreover, by plotting a few ordinates of the curve for the result the practical range of numbers of *B. coli* which may be in the sample is readily determined, in the case of the single large sample. But in the case of the several small samples, no such estimate of the precision of the average result is possible.

The only method of obtaining a good average result from several samples is, therefore, that of mixing the sample before the analysis, thus making a composite sample from which many tubes may be inoculated. And the precision which may be obtained in the result from this composite sample is limited only by the size of the sample.

VARIOUS METHODS OF MAKING DILUTIONS

By an extension of the mathematical analysis (too lengthy a development to be included here), it may be shown that the following methods of obtaining 0.1 c.c. of the sample allow the same probability of drawing some *B. coli* in that 0.1 c.c.: (1) taking, by means of a pipette, exactly 0.1 c.c. of the sample; (2) mixing exactly 1 c.c. of the sample with 9 c.c. of sterile water, and taking exactly 1 c.c. of the mixture; (3) mixing exactly 10 c.c. of sample with 90 c.c. of sterile water and taking exactly 1 c.c. of the mixture. In practice, however, the different facilities of error involved in measuring out the different quantities of sample and water, may upset this balance of probability.

EXPERIMENTAL

To test the fundamental general formulae given by theory, two sets of experiments were made.

With Seeds.—A certain number of white seeds (representing *B. coli*) were mixed with 100 volumes of black seeds (representing the sample). From this mixture were then drawn a certain number of volumes, and at each draw presence or absence of white seeds noted and recorded. These volumes were then replaced in the container, the whole thoroughly mixed again, and the first procedure repeated.

This was continued until 50, 100, or 200 trials had been made, as indicated in Table 3.

This procedure corresponds to the analysis of 50, 100, or 200 samples, each of which contained the same number of *B. coli*. It is to be noted that the conditions obtaining in the drawing of the several

volumes from each sample were exactly the same as those which obtain in actual analysis of a water sample: there was no replacement after each separate draw.

Table 3 shows the results obtained, together with the theoretical results which should have been obtained according to the general formulae of Case 2.

The agreement between experiment and theory is remarkably close, if the small number of trials is considered, despite the fact that the

TABLE 3
SHOWING THE NUMBER OF TIMES CERTAIN RESULTS OCCURRED IN THE EXPERIMENT, AND THE MOST PROBABLE NUMBER OF TIMES THESE RESULTS SHOULD HAVE OCCURRED ACCORDING TO THEORY

Result	40 White Seeds in 100 Volumes (200 Trials)		160 White Seeds in 100 Volumes (100 Trials)		160 White Seeds in 88 Volumes (150 Trials)*		240 White Seeds in 100 Volumes (50 Trials)		320 White Seeds in 100 Volumes (50 Trials)	
	Experi- ment	Theory	Experi- ment	Theory	Experi- ment	Theory	Experi- ment	Theory	Experi- ment	Theory
0/1	147	134	16	20	26	24	6		4	2
1/1	53	66	84	80	124	126	44	46	46	43
0/2	107	90	3	4	6	4	1	
1/2	80	89	26	32	49	41	9	8	6	4
2/2	13	21	71	64	95	105	41	42	43	46
0/5	41	27								
1/5	61	66	1	1	...	1				
2/5	59	65	2	5	5	5				
3/5	26	32	19	21	22	24	3	3	2	1
4/5	12	8	40	41	73	60	15	16	10	9
5/5	1	1	38	33	50	61	32	31	38	40
0/10	4	4								
1/10	21	18								
2/10	41	40								
3/10	55	52								
4/10	45	43	...	1	2					
5/10	23	27	2	3	2					
6/10	10	11	10	9	12	8	1			
7/10	...	3	16	20	34	23	3	2		
8/10	1	1	30	30	41	43	6	9	4	3
9/10	31	27	43	49	17	19	13	14
10/10	11	11	16	25	23	20	33	33

* The theoretical calculations for this case are based on "180 white seeds in 100 volumes," a condition which is roughly equivalent to that of "160 white seeds in 88 volumes." Theory gives practically identical probabilities for these two conditions.

It must be remembered that even in these experiments Chance is operating, and exact correspondence between Experiment and Theory is not to be expected with only one short series of trials. But with a greater and greater number of trials, the percentage "error," or difference between Experiment and Theory, may be expected to become correspondingly less.

theoretical results were calculated on the assumption of replacement after each separate draw. It is evident that, for all practical purposes, this assumption may be disregarded, and the general formulae applied directly to the fermentation-tube results which may be obtained in the laboratory.

With Bacillus coli.—A large bottle was almost filled with about 8,000 c.c. of normal salt solution, sterilized, and after it had been

cooled, carbon-dioxid was blown through it for a few minutes. (It was feared that practical absence of this gas might prove destructive to the organism to be added.)

A twenty-four-hour bile culture of *B. coli* was filtered through sterile filter-paper, and a few drops of the filtrate added to the solution, which was then well mixed and placed in the dark.

After twenty-four hours several bile tubes were inoculated with 1 c.c. of the solution, which, after another twenty-four hours, gave results indicating the number of *B. coli* in the sample to be such that no dilutions would be required for analysis by the fermentation test.

The sample after thorough mixing, was then tested as follows: With a 10 c.c. pipette graduated in tenths, 10 c.c. of the sample (8,000 c.c. in quantity) was withdrawn, and 1 c.c. inoculated into each of ten Durham tubes containing lactose peptone bile. The ten tubes were held together as a unit, being contained in a small zinc box into which they fitted.

After ten such boxes of tubes had been inoculated in this manner, the sample was well shaken, and another ten boxes of tubes inoculated; and so on, until 76 boxes of tubes, or 760 tubes, each contained 1 c.c. of the sample.

After forty-eight hours' incubation at 37 C., the number of positives were distributed as follows:

First 100 tubes	71 positives
Next 100 tubes	79 positives
Next 100 tubes	77 positives
Next 100 tubes	79 positives
Next 100 tubes	78 positives
Next 100 tubes	85 positives
Next 100 tubes	82 positives
Next 60 tubes	46 positives (77 per cent.)

Thus the total number of positives was 597* out of 760 inoculated. The most probable number of *B. coli* per 100 c.c. (capable of fermenting the media), in the sample, is then given by the solution of the equation:

$$1 - .99^x = \frac{597}{760}$$

and, this being solved, $x=153$, to the nearest unit.

Now, if the number of *B. coli* in the sample was really about 153 per 100 c.c., the number of times the particular results "10/10 in

* Every tube of the 597 positive tubes contained a large amount of gas, except one. This tube contained only about 2 percent of gas, but was recorded as positive. Seventy-two hours' incubation gave no further positives.

1 c.c." "9/10 in 1 c.c.", etc., occurred, should approximate the number of times these results should occur according to calculation by the general formulae of Case 2, with $x=153$. Thus, the result "10/10 in 1 c.c." should occur $76(1-.99^{153})^{10}=6.764$, or 7 times, to the nearest unit.

These calculations, together with the number of times the various results did actually occur, are given below:

Result	Experiment	Theory
$\frac{4}{10}$	6	0
$\frac{5}{10}$	4	3
$\frac{6}{10}$	7	8
$\frac{7}{10}$	15	17
$\frac{8}{10}$	27	23
$\frac{9}{10}$	16	18
$\frac{10}{10}$	7	7
	<hr/> 76	<hr/> 76

Taking only the results given by the first five tubes, the first two tubes, the first tube, of each box of ten tubes, we have the actual and calculated results as follows:

Result	Exp'm't	Theory	Result	Exp'm't	Theory	Result	Exp'm't	Theory
$\frac{5}{5}$	26	23	$\frac{2}{5}$	42	47	$\frac{1}{1}$	21	16
$\frac{4}{5}$	25	31	$\frac{1}{2}$	32	26	$\frac{0}{1}$	55	60
$\frac{3}{5}$	21	17	$\frac{0}{2}$	2	3			
$\frac{2}{5}$	4	5						
$\frac{1}{5}$	0	0						

The close correspondence between the experimental and theoretical results, afford an excellent verification of the "most probable number," 153 *B. coli* per 100 c.c., which was obtained by the other formula, and which was in no way dependent upon the formulae used in these latter calculations.

These experiments prove beyond doubt that the general formulae given in the early part of this paper are applicable to the general problem of numerical interpretation of fermentation-tube results. In all these experiments the procedure adopted was strictly analogous to that obtaining in laboratory practice; yet, despite the assumptions involved in the theoretical calculations, as regards quantity of sample and replacement after every draw, the agreement between experiment and theory is all that could be desired.

One particular point, which is especially emphasized by the results of these experiments, should be noted, namely, the persistence with which results of small probability (occurring with small frequency) do

occur. In nearly all the series of experimental results given in this paper, the number of results of small frequency check very closely the number indicated by theory. The conclusion is obvious: The odd and infrequent result is bound to occur with its allotted frequency.

SUMMARY

The frequency of the appearance of the fermenting organism in the volume drawn from the sample for the test is an exponential function of the number of such organisms in the sample.

Every fermentation-tube result, whether simple or compound, corresponds to one most probable number of organisms, and this number demands the same consideration as does the most probable number of bacteria corresponding to the plate-count in the estimation of other groups of bacteria. A simple method is available by which this most probable number of organisms may be calculated.

Odd results, such as "anomalies," are sure to occur with their theoretical frequency, and should not be thrown out of the record, but preserved and given their numerical interpretations.

Methods are available by which some knowledge of the degree of precision of a result may be obtained. In consequence, different results may be compared, the significance of the difference between certain results may be determined, and sampling may be so conducted as to give, in the result, any degree of precision desired.

Because of the difficulty of obtaining a trustworthy average of fermentation-tube results, the results should be expressed in full, together with a statement of the "most probable number" of organisms in the sample.

Calculation indicates that the precision of the fermentation-tube method compares not unfavorably with that of the plate method, of analysis.

The current methods of making dilutions should all give the same results, so far as chance distribution of the organisms is concerned.

Both calculation and experiment show that, for all practical purposes, the general formulae given are applicable to the problem of numerical interpretation of fermentation-tube results obtained in the laboratory.* For the severe conditions involved in the method of analysis prescribed by the U. S. Treasury Standard for waters sup-

* The writer has prepared a table which contains logarithms for the rapid calculation of "most probable numbers," values of the ordinates of the curves for the results $N/1$, $N/2$, $N/3$, $N/5$ and $N/10$, etc., copies of which he will be glad to furnish upon request. Address 9 St. James St., Montreal.

plied by common carriers, special formulae are necessary, which are given. (The sample should always be thoroughly mixed before testing. If not, the Probabilities, as herein developed, will not hold.)

For the proof that various methods of making dilutions give the same probability; for the methods of summing the ordinates of the curves; for the formula giving the probability of obtaining a specified number of organisms in the volume drawn, as well as for much helpful criticism and suggestion, the writer is greatly indebted to Professor Wm. D. Cairns, Associate Professor of Mathematics, Oberlin University, Ohio.

ADDITIONAL NOTE ON A METHOD OF EMPLOYING A LARGE NUMBER OF TUBES IN THE FERMENTATION TEST

It is evident from the foregoing study, that to attain even a fair degree of precision with the fermentation test, several tubes must be used at each dilution. But the use of several tubes is attended by considerable inconvenience when they are handled in the ordinary manner, and until this inconvenience is largely obviated by means of some improvement in apparatus, increase in precision in routine work will perhaps not become very general. For two years the Laboratory of the Board of Health of the Province of Quebec has employed the following system of analysis on all ordinary samples of water where the field survey does not reveal heavy pollution:

(1) Ten cubic centimeters of the sample are withdrawn by means of a straight-walled 10 c.c. pipette, and mixed with 90 c.c. of sterile water.

With another 10 c.c. pipette, 10 c.c. of this mixture are withdrawn and 1 c.c. delivered into each of ten Durham bile tubes. This gives ten tubes with 0.1 c.c. of the sample in each.

(2). With the other pipette, 10 c.c. of the sample are withdrawn and 1 c.c. delivered into each of ten tubes.

(3). With the same pipette 10 c.c. of the sample are delivered into each of two large tubes.

Results from this system will be of the form:

"A/2 in 10 c.c., B/10 in 1 c.c., C/10 in 0.1 c.c."

Such a procedure may appear laborious, but by means of a simple contrivance for handling the tubes, the labor involved is very little more than that in using only two tubes at each dilution in the ordinary manner. This contrivance consists simply of a small zinc box, into which ten Durham tubes fit rather loosely. The outer tube of the fermentation tube is a large, flat-bottomed specimen tube; the inner tube is of the same pattern but smaller.

When preparing the apparatus, the outer tubes are placed in position in the boxes, and filled to the proper height with the medium by moving from tube to tube a large cylindrical funnel provided with a rubber tube and pinch-cock. A large number of tubes may be filled very rapidly in this manner. The inner tubes are then dropped into place, and the cover of the box, into which has been placed a strip of cotton, is fitted over the tops of the tubes and secured by a rubber band, as shown in the accompanying figure. The one strip of cotton thus serves as a common plug for all the tubes, being held firmly in place by the pressure of the rubber band. If the tubes are not to be used at

once, they are wrapped in ordinary wrapping paper (which may be obtained of the desired width), before securing with the rubber band. The boxes are then sterilized in the ordinary way.

When using the tubes, the paper wrapper is removed, the cover lifted, and by means of a straight-walled 10 c.c. pipette, 10 c.c. of the sample are withdrawn and 1 c.c. of this delivered into each of the ten tubes in the box by simply moving the pipette from tube to tube. The cover is then dropped into

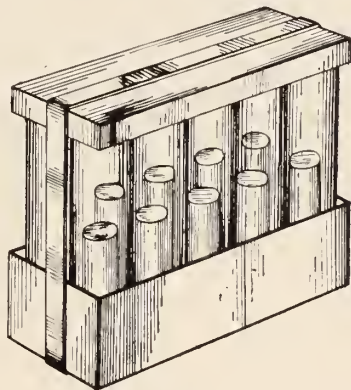


Figure 4

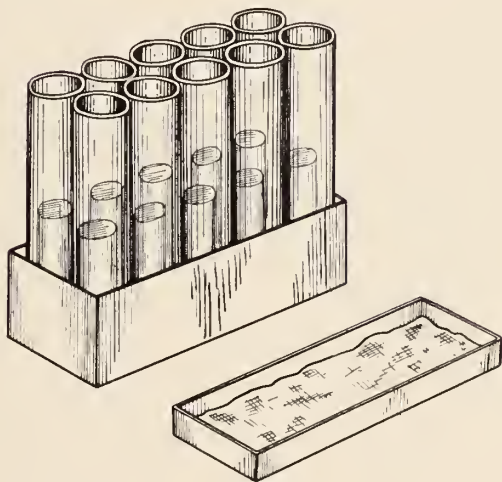


Figure 5

place, the rubber band slipped around the whole, and the box marked with the number of the sample and the number of the dilution. The box is then ready for the incubator, for re-wrapping with the paper is quite unnecessary.

The advantages of this method are many:

(1). A more reliable result (greater precision) is obtained with the large number of tubes, and with very little extra labor.

(2) All tubes at the same dilution for the same sample are held together as a unit, reading of the results being thus rendered a very simple operation.

(3) The box is marked, the inconvenience attending the marking of each individual tube being thus obviated.

(4). The shape of the boxes permits very easy handling, and very compact packing in the incubator, for they may be packed one upon the other.

(5) In experimental work, some such device is practically indispensable. For instance, in the experiment described in the foregoing paper in which 760 tubes were inoculated, each with 1 c.c. of the sample, it would have been quite out of the question to have attempted the inoculation of such a large number of tubes in the usual manner of handling each tube separately.

Two years' experience with this method of employing large numbers of tubes in the fermentation test have amply demonstrated the facility with which the apparatus may be prepared and manipulated, and the greater satisfaction to be derived from the corresponding increase in precision obtained by its use.

COMPLEMENT FIXATION IN HOG CHOLERA *

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During the summer of 1914, while working on the etiology of hog-cholera, we found it desirable to try the complement-fixation test, using as antigen a certain micro-organism which had been isolated. These tests were not successful. This organism had been isolated from the mesenteric glands of acute cholera hogs. The pathologic changes in such glands were frequently so marked that it seemed highly probable that the cholera virus was present in them to a marked degree. Therefore, one of us (Healy) attempted the preparation of an antigen by extraction of these glands in the following manner:

The mesenteric glands, which were enlarged, dark in color, and congested, were obtained from three acute cholera hogs which had been bled for virus. The glands were dissected from the surrounding tissues and ground with sterile sand. The resultant mass was then placed on ice over night at a temperature of 4 C. On the following day, a 1 percent neutral glucose beef broth was added to the ground glands in the proportion of one part by weight of unground glands to ten parts by weight of glucose broth, and the mixture again placed on ice at 4 C. for eight days. At the end of this period, a portion of the mixture was passed through a Royal Berlin porcelain, open-topped, Pasteur-Chamberland filter—bougie E. & A., 1802. The filtrate, of a pale amber color and perfectly clear, was used, unheated, as antigen. All manipulations were performed as aseptically as possible. The hog serum used was an amber-colored serum obtained from a hyperimmune hog. This serum had been on ice for four months. The first complement-fixation test resulted as follows:

Antigen in c.c.	Normal Salt Solution in c.c.	Immune Serum in c.c.	Complement in c.c.	Hemolysin in c.c.	Red Blood Corpuscles in c.c.	Hemolysis
0.10	1.5	0.05	0.045	0.15	0.5	Complete
0.13	1.5	0.05	0.045	0.15	0.5	None
0.15	1.5	0.05	0.045	0.15	0.5	None
0.17	1.5	0.05	0.045	0.15	0.5	None
0.20	1.5	0.05	0.045	0.15	0.5	None
0.25	1.5	0.05	0.045	0.15	0.5	None
0.30	1.5	0.05	0.045	0.15	0.5	None

The antigen, immune serum, and complement were mixed and placed at 37 C. for one hour, whereupon the hemolysin and corpuscles were added and the whole incubated at 37 C. for two hours.

* Received for publication May 10, 1915.

The test for complement absorption was negative. The tests for antigen, serum, complement, and lysin resulted as follows:

Antigen in c.c.	Normal Salt Solution in c.c.	Immune Serum in c.c.	Complement in c.c.	Hemolysin in c.c.	Red Blood Corpuscles in c.c.	Results
0	1.5	0.05	0.045	0.15	0.5	Complete
0.65	1.5	0	0.045	0.15	0.5	Complete
0.65	1.5	0.05	0	0.15	0.5	None
0.65	1.5	0.05	0.045	0	0.5	None

The test was repeated on the following day with the same results.

After this antigen had stood on ice at a temperature of 4 C. for twenty-three days, it was again tested with the following result, the test being made in exactly the same way as before:

Antigen in c.c.	Hemolysis
0.10	Complete
0.13	Complete
0.15	Nearly complete
0.17	Partial
0.20	Trace
0.25	None
0.30	None

The antigen, now passed through an imported Pasteur-Chamberland "F" bougie, lost the power to fix complement in the strength shown in the preceding table.

It is of interest that the strength of the antigen diminished with time, and, indeed, when tested on the twenty-sixth day, gave a negative reaction throughout. This alteration of antigen was possibly due to slight micro-organic growth. It is of considerable interest also that the imported "F" bougie removed the antigen altogether.

There can be no doubt that a reliable complement-fixation test for hog-cholera would be of value, not only as an aid in the study of this disease, but also as a means of diagnosis in those cases of chronic hog-cholera in which malnutrition is the only symptom. Possibly also such a test might be used successfully in the standardization of hyperimmune serum and the demonstration of immunity in a given hog.

On further study of this test, we were impressed with the following facts: That the mesenteric glands used must show distinct pathologic changes, indicated by increase in size, darkness of color, and congestion; that the suspension of such ground glands in the 1 percent glucose beef broth must remain at a temperature of 4 C. for at least eight days before the antigen develops; and that all manipulations must be as aseptic as possible.

Washed sheep blood corpuscles were used in the tests. The blood was drawn from the jugular vein through a large hypodermic needle and allowed to flow into a sterile bottle containing glass beads. The blood was immediately and thoroughly shaken for five to ten minutes to defibrinate it. It was then filtered through sterile cotton into 100 c.c. centrifuge tubes, about 20-30 c.c. of blood being placed in each tube. The tubes were next filled with sterile, normal salt solution and centrifugated at 4,000 revolutions for twelve minutes, the supernatant liquor pipetted off, and the operation repeated. This washing was repeated four times, after which a 2 percent suspension of the corpuscles in normal salt solution was prepared. We used 0.5 c.c. of such a suspension in the tests described.

The clear serum of a rabbit which had received repeated injections of sheep corpuscles, washed as above, was used for hemolysin. Ten cubic centimeters of washed sheep corpuscles were mixed with an equal quantity of sterile, normal salt solution, and injected intraperitoneally into the rabbit at eight-day intervals. The rabbit received six such injections. Eight days after the last injection, a small quantity of blood was withdrawn from an ear vein, and after the clotting of the blood the serum was tested. If the serum was of sufficient strength, the rabbit was bled and the clear serum obtained from the clot. This serum was heated to 56 C. for thirty minutes to destroy complement, and then was preserved by the addition of 0.2 percent carbolic acid, and kept on ice. In our tests, 1 c.c. of the inactivated rabbit serum, added to 25 c.c. sterile, normal salt solution, was used as hemolysin. This mixture was titrated against 0.5 c.c. of the 2 percent washed blood corpuscle suspension and a quantity of complement of known strength. Thus:

Hemolysin in c.c.	Normal Salt Solution in c.c.	Complement in c.c.	Red Blood Corpuscles c.c.	Hemolysis
0.01	1.5	0.03	0.05	None
0.02	1.5	0.03	0.05	Partial
0.03	1.5	0.03	0.05	Complete
0.04	1.5	0.03	0.05	Complete
0.05	1.5	0.03	0.05	Complete
0.06	1.5	0.03	0.05	Complete

The titer of this hemolysin was 0.03, and the dose (five times the titer) was 0.15 c.c. This hemolysin was used in the first tests. The second hemolysin gave a titer of 0.02, the dose being 0.10 c.c.

Normal guinea-pig serum furnishes the strongest and most stable complement. In this experiment, the guinea-pig was bled from the throat and the blood allowed to flow through a sterile funnel into a sterile 50 c.c. centrifuge tube, where it rapidly clotted. The clot was separated from the side of the tube with a sterile glass rod and was then placed at 37 C. for thirty minutes to one hour. The separated serum was placed in small tubes and centrifugated until clear. The serum was then mixed with twice its volume of sterile, normal salt solution and titrated against a known quantity of washed sheep corpuscles, and a quantity of inactivated, sensitized rabbit serum of known strength. Thus:

Complement in c.c.	Normal Salt Solution in c.c.	Hemolysin in c.c.	Red Blood Corpuscles in c.c.	Hemolysis
0.01	1.5	0.15	0.5	None
0.02	1.5	0.15	0.5	Nearly complete
0.03	1.5	0.15	0.5	Complete
0.04	1.5	0.15	0.5	Complete
0.05	1.5	0.15	0.5	Complete
0.06	1.5	0.15	0.5	Complete

The titer of this complement was 0.03, and the dose (1.5 times the titer) was 0.045 c.c. The complement, if kept on ice at about 6 C., retained its strength for several days. The complement had to be titrated very day, however, as it generally lost its strength, and that sometimes rapidly.

Eighteen grams of selected mesenteric glands from acute cholera hogs bled for virus, were thoroughly ground with sterile sand; 180 gm. of neutral 1 percent glucose beef broth were added and the whole placed at 4 C. At the end of forty hours, a portion of this suspension was passed through an ordinary white filter paper, and tested, unheated, with wholly negative result. The hog serum was amber serum obtained from a hyperimmune hog. This serum had been on ice for ten months.

At the end of five days, another portion of this suspension was passed through an ordinary white filter paper and tested, again with negative result. The test for complement absorption was negative.

At the end of eight days another portion of this suspension of mesenteric glands was passed through an ordinary white filter paper. The filtrate was quite clear and of an amber color. This antigen was tested, unheated, with three different immune sera in the same way as that described. Serum 1 was from a hyperimmune hog, and had been on ice for ten months; Serum 2 was from another hyperimmune hog, and had been on ice eighteen days; Serum 3 was from a hog on a farm where all hogs were immune. The result was as follows:

Antigen in c.c.	Hemolysis with Serum 1	Hemolysis with Serum 2	Hemolysis with Serum 3
0.10	None	Complete	Complete
0.13	None	None	None
0.15	None	Trace	None
0.17	None	Trace	Trace
0.20	Trace	Trace	Trace
0.25	Trace	Complete	Trace
0.30	Complete	Complete	Complete
0.0	Complete	Complete	Complete

The test for complement absorption was negative.

To obtain absolutely normal hog serum is, at the present time, most difficult. While it is true that there are some farms on which hog-cholera as yet has not appeared, there is a strong probability that the hogs on these farms possess a certain degree of immunity, either inherited or acquired. After careful search, we selected three hogs which appeared as normal as one could reasonably expect to find. Clear, amber serum was obtained from each of these hogs

and tested according to the scheme outlined with the following results. The immune Serum I was used as a check, as were also normal rabbit serum and normal cow serum.

Antigen in c.c.	Hemolysis with Normal Hog Serum 1	Hemolysis with Normal Hog Serum 2	Hemolysis with Normal Hog Serum 3	Hemolysis with Immune Serum 1	Hemolysis with Normal Cow Serum	Hemolysis with Normal Rabbit Serum
0.10	Complete	Complete	Complete	Nearly complete	Complete	Complete
0.13	Nearly Complete	Complete	Complete	Nearly complete	Complete	Trace
0.15	Partial	Complete	Nearly complete	None	Complete	None
0.17	Trace	Partial	Trace	None	Complete	Complete
0.20	None	Partial	None	None	Complete	None
0.25	None	None	None	None	Complete	None
0.30	None	None	None	None	Complete	None
0.00	Complete	Complete	Complete	Complete	Complete	Complete

In this series the normal rabbit serum was a disturbing element. This rabbit serum, however, reacted in an irregular manner throughout; in the following tests, in which the quantity of antigen was uniform (0.1 c.c.) and the quantity of serum varied, the rabbit serum yielded more consistent results:

Quantity of Serum in c.c.	Hemolysis with Normal Hog Serum 1	Hemolysis with Normal Hog Serum 2	Hemolysis with Normal Hog Serum 3	Hemolysis with Normal Rabbit Serum	Hemolysis with Immune Serum 1
0.005	Trace	None	Partial	Nearly complete	Partial
0.008	Complete	Complete	Complete	Partial	None
0.01	Complete	Partial	Complete	Trace	None
0.02	Complete	Complete	Complete	Complete	None
0.03	Complete	Complete	Complete	Complete	Nearly complete
0.04	Complete	Complete	Complete	Nearly complete	Complete
0.05	Complete	Complete	Complete	Nearly complete	Nearly complete
0.10	Complete	Complete	Complete	Complete	Complete
0.20	Complete	Complete	Complete	Complete	Complete

A thorough search of the literature has failed to afford a record of complement fixation in hog-cholera. It must not be understood that we claim to have obtained a specific antigen for the hog-cholera antibodies. It will require considerable work either to prove or disprove the specific nature of this antigen which we have developed. We have obtained an antigen which shows striking differences in its reaction toward normal hog, rabbit, and cow sera, and hyperimmune hog serum. This antigen is not present in the freshly prepared extract of mesenteric glands, but requires a definite period for development; it is not removed from such an extract by passage through an ordinary porcelain filter, but is removed by passage through the "F" bougie; finally it gradually disappears from the extract.

In the Wassermann reaction there is no analogy between the extract of syphilitic liver and true antigen, and yet the results of

this method of diagnosis have, on the whole (with certain reservations), been satisfactory.

We are continuing this work, especially in the direction of improving the method of preparing the antigen, and of increasing its sensitiveness.

THE BACTERIOLOGY OF ULCER OF THE STOMACH AND DUODENUM IN MAN*

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(WITH PLATES 4 AND 5)

It is recognized that hemorrhage of the stomach with or without visible ulceration occurs rather commonly during the late stages of severe streptococcal infections in man and animals. The cause of the gastric lesions in these cases usually is thought to be the profound toxemia, in spite of the fact that many observers have demonstrated the presence of streptococci or diplococci in the lesions in the mucous membrane. The cause of the usual ulcer of the stomach in man, which tends to persist and which occurs commonly in otherwise healthy individuals, the usual symptoms or other evidences of infection being slight or wholly absent, is not clearly established. The presence of pyorrhea, as noted by Bolton¹ and others, of blind abscesses about the roots of teeth, of chronically infected tonsils and sinuses about the head; the occurrence of acute attacks of such infections, especially streptococcal, often a week or ten days previous to acute ulceration and previous to exacerbation of symptoms in chronic ulcer; the aggravation of symptoms in chronic duodenal ulcer during the months when throat and other streptococcal infections are particularly prevalent, as emphasized by Moynihan²; the improvement in symptoms following eradication of foci of infection, as noted especially by Billings;³ and the fact that streptococci when of a certain grade of virulence, quite irrespective of their original source, are prone to localize in the mucous membrane of the stomach and duodenum of animals following intravenous injection, producing ulcers which resemble those in man, as shown by Rosenow⁴—all suggest strongly that a localized streptococcal infection may be an important factor in the original production of the ulceration.

* Received for publication May 12, 1915.

1. Ulcer of Stomach, 1913.

2. Duodenal Ulcer, 1913.

3. Jour. Am. Med. Assn., 1914, 63, p. 399.

4. Jour. Am. Med. Assn., 1913, 61, p. 1947.

Heretofore, the cultures of ulcers of the stomach have been made chiefly after death and from the floor of the ulcer, and not usually from the emulsified tissues remote from the ulcerated area. The results, as should be expected, have been so unsatisfactory as to lead to a general belief that bacteriologic examination of ulcers as they occur in man cannot be made with any degree of satisfaction. However, a systematic bacteriologic study of the emulsified tissues surrounding the ulceration, removed during life at operation, should yield more trustworthy results.

In this paper, we wish to record the results of cultures and of the histologic examination of the tissues in a series of ulcers and lymph glands draining the ulcers, excised at operation. We wish here to express our appreciation of the opportunity afforded us in the willing co-operation of Dr. W. J. Mayo, Dr. C. H. Mayo, Dr. E. S. Judd, Dr. D. C. Balfour, Dr. A. J. Ochsner, Dr. A. D. Bevan, Dr. L. L. McArthur and Dr. C. H. McKenna.

TECHNIC

The ulcers and other tissues were excised under strictly aseptic precautions, covered at once with sterile gauze, and cultures made as soon as possible thereafter, usually within one to three hours. After some of the material from the floor of the ulcer, in suitable cases, had been withdrawn into a sterile pipette (for control cultures) and the surface of the ulcer thoroughly washed in running, sterile water, approximately one-half of the ulcer was excised with a small, sterile scalpel, the incisions being made from the normal tissue toward the center of the ulcerated area. The excised part was again washed thoroughly in sterile water or salt solution and the emulsion prepared in the sterile air chamber directly if the size of the tissue was small, or after the surface had been sterilized with passing through a very hot Bunsen flame or with a searing blade, if the piece of tissue was large enough. Cultures were made in some instances in the same way from the adjacent normal wall and in a number of instances from the indurated area far removed from the ulcerated surface and from the overlying mucous membrane. The lymph glands and other tissues were treated in a similar manner.

The emulsions were prepared in dextrose broth or NaCl solution, and the cultures made chiefly in tall columns (9-12 cm.) of dextrose agar and broth, with and without the addition of sterile ascites fluid, and on Loeffler's blood serum and ascites agar slants, as described by Rosenow.⁵

The portion of the ulcer saved for microscopic study was fixed in alcohol or 10 percent formalin, imbedded in paraffin, stained with hematoxylin and eosin and for bacteria by the Gram-Weigert method. In this method complete decolorization was not practiced, because it was found that streptococci present were very easily decolorized; hence decolorization was carried to a pale blue only.

Cultures have been made from ulcers or regional lymph glands or from both in thirty-two cases. In fifteen the ulcer was situated in the pyloric end of the stomach, in five at the lesser curvature, in three in the fundus, and in twelve in the duodenum. In three cases there were both ulcer of the stomach and duodenum. Chronic appendicitis was associated with ulcer in seven of the cases, cholecystitis in five, and pancreatitis in three. Appendicitis, ulcer, and cholecystitis coexisted in two cases; ulcer, cholecystitis, and pancreatitis in one case. The age of the patients ranged from twenty to seventy-two years. The duration of the symptoms of ulcer at the time of operation ranged from six months to twenty-three years.

Cultures were made from the wall of ulcers in twenty-four cases. In these, streptococci were isolated in varying numbers (1-5000 colonies) in pure culture from nine ulcers, and in mixtures in all but one of the remaining fifteen. If judged by the numbers of streptococci found in the portion of the ulcers sectioned, it is certain that the number of colonies obtained in the cultures did not represent the actual number present; either some were dead to begin with, or from long residence in the tissue failed to grow in the new environment, or many were killed in the sterilization of the surface. In two cases of duodenal ulcer (one and twelve years old,) in which the ulcers were so situated as to make their total removal impossible, the usual streptococcus was isolated from a thin layer of the inflamed peritoneum directly over the ulcer, and in one from the hyperemic parietal peritoneum directly opposite the ulcer (Cases 112, 134). In the former, diplococci were demonstrated in the tissue (Fig. 8). In the latter, this was not attempted. Non-hemolysing staphylococci were isolated from ten cases, but never in pure form. *Staphylococcus aureus* was found in one ulcer, but not in pure form (Case 902). *Bacillus welchii*, in small numbers, was found in the ulcers or glands in four cases. They developed only in those tubes containing a large amount of the emulsion. A gram-positive bacillus, probably belonging to the subtilis group, was found in six cases, and a large, unidentified, gram-negative bacillus in three. The colon bacillus was found in rather large numbers in two cases after death, and in small numbers in a duodenal ulcer in one case during life. Yeast cells were isolated in large numbers from three ulcers, and in small numbers from one. A few sarcinae were grown in one case, and diphtheroid bacilli in three cases.

TABLE 1
SUMMARY OF FACTS AND RESULTS OF CULTURES IN GASTRODUODENAL ULCER

Case	Sex and Age	Probable Age of Ulcer	Location and Character of Ulcer	Result of Cultures
1	F., 25	3 years	Puckered ulcer of duodenum 2 cm. from the pylorus (0.5 x 0.6 cm.)	100 colonies of streptococci; a few staphylococci
2	F., 40	10 years	Constricted ulcer, anterior superior wall, 2 cm. above pyloric ring, 0.5 cm. in diameter with clean base and infiltrated margin	A few colonies of large, gram-staining bacilli and staphylococci, and 280 colonies of streptococci
B	F., 28	5 years	Ulcer of stomach causing hour glass constriction	Moderate number of streptococci and staphylococci
D	M., 41	15 years	Indurated, puckered, deep ulcer of pyloric ring	Many colonies of yeast, gram-negative bacilli, streptococci, and staphylococci
E	Indurated ulcer of stomach..	Streptococci and a few staphylococci, and a large gram-negative bacillus
F	F., 26	6 weeks	Ulcer of duodenum 2 cm. beyond pyloric ring	200 colonies of streptococci; a few colonies of staphylococci and saprophytic bacilli
J	M., 54	6 years	Indurated ulcers in stomach and duodenum	Pure culture of streptococci
23 99	Indurated ulcer of pylorus...	Streptococci, staphylococci, and large number of colon bacilli (cultures made after death)
773	M., 53	3 years	Indurated duodenal ulcer adherent to gall-bladder	1500 colonies of staphylococci, and 20 colonies of streptococci
779	F., 43	12 years	Indurated healing ulcer of duodenum	180 colonies of staphylococci; 8 colonies of streptococci
849	M., 26	7 years	Indurated ulcer of duodenum	One colony of streptococci from gland draining ulcer
860	M., 50	A number of hard scars in pylorus and duodenum. Indurated undermined ulcer of pylorus (1 x 0.6 cm.)	Pure culture of streptococci from depths of ulcer; gland sterile
870	M., 65	3 months	Large, markedly indurated ulcer, extending half way around pylorus	Staphylococci, streptococci, and a few colonies of <i>Bacillus Welchii</i>
885	3 weeks	Acute perforating ulcer of stomach	5000 colonies of a distinct, green-producing streptococcus in pure culture
886	M., 46	Chronic ulcer of stomach....	Lymph gland cultured: two colonies of streptococci, and ten of <i>Bacillus Welchii</i>
893	M., 47	12 years	Indurated ulcer of duodenum (2.5 cm.), perforated into head of pancreas	2000 colonies of yeast, and a few sarcinae and streptococci from ulcer; from gland, streptococci only; adjacent normal mucous membrane sterile
902	M., 51	4 years	Very large, markedly indurated, crater-like ulcer of pylorus (2 x 2.5 cm.)	340 colonies streptococci, 120 colonies <i>Staphylococcus aureus</i> , and 50 colonies of a diphtheroid bacillus
904	F., 20	A few days	Small, numerous, punctate ulcers of stomach, chiefly of fundus	Large number of green-producing streptococci with marked involution forms, a few staphylococci and colon bacilli (cultures made six hours after death)
909	F., 42	Ulcer of stomach.....	Cultures of gland from pylorus remained sterile
947	6 months	Adherent ulcer of duodenum	Gland shows approximately 4200 colonies of green-producing streptococci in pure culture
31	F	Many years	Markedly indurated ulcer of pylorus, margin abrupt, hard, and crater-like (3 x 2.5 cm.)	<i>Streptococcus viridans</i> , gram-positive and negative, bacilli, and staphylococci from floor of ulcer; streptococci from depths of indurated wall and from adjacent lymph gland

TABLE 1—Continued

Case	Sex and Age	Probable Age of Ulcer	Location and Character of Ulcer	Result of Cultures
52	F., 62	10 days	Five ulcers, three in the duodenum and two in pylorus	6000 colonies streptococci, moderate number of colon bacilli, and a few staphylococci (culture made soon after death)
63	Many years	Indurated ulcer of lesser curvature	Gland cultured: spore-bearing bacilli, streptococci, and staphylococci
69	Indurated ulcer, posterior wall near lesser curvature	From cultures from floor of ulcer, a few staphylococci; from emulsion of wall, two colonies of streptococci and gram-staining bacilli
82	Large indurated ulcer of lesser curvature	Cultures from two glands negative
83	M., 69	11 years	Ulcer of duodenum perforating into pancreas	Cultures from gland negative
104	M., 54	10 years	Large ulcers, one of lesser curvature and one of duodenum	From duodenal ulcer, a few colonies of colon bacilli, <i>Bacillus Welchii</i> , staphylococci, and streptococci. From gastric ulcer, large number of yeast colonies in upper portion of tube.
112	F. 51	1 year	Subacute ulcer of duodenum; visceral and parietal peritoneum markedly congested	From parietal peritoneum, five colonies of streptococci; from visceral peritoneum directly over ulcer, moderate number of streptococci, and a few colonies of slender, gram-staining bacilli
120	M., 55	7 years	Ulcer of duodenum just beyond pylorus, involving almost entire circumference	Gland gave 18 colonies of streptococci
126	M., 20	10 years	Ulcer of duodenum 2 cm. beyond pyloric ring with recent perforation	Gland gave pure culture of streptococci
134	M., 29	12 years	Duodenal ulcer just beyond pylorus	Peritoneum directly over ulcer gave pure culture streptococci

The results of the cultures in the broth were usually the same as those in the agar, altho in some instances the former were positive when the latter were negative. The anaerobic cultures on blood agar and Loeffler's serum slants were nearly always sterile. A number of times marked odor-producing bacilli were isolated but these were not identified.

Lymph glands, varying in size from 0.5 to 1.5 cm. in diameter, draining the ulcers, were cultured in eleven cases. Of these, five appeared to be sterile, four yielded streptococci in pure culture, two streptococci together with a few colonies of *Bacillus welchii*. Colon bacilli were not found in any of the glands. The glands which proved sterile were all from cases of ulcer of long standing in which no exacerbation of symptoms occurred shortly before surgical intervention.

Both the ulcer and the adjacent lymph glands were cultured in two cases (89 and 31). The glands in both yielded pure cultures of streptococci: the ulcer in Case 89 gave these together with yeast and sarcinae; in Case 31, the ulcer gave *Streptococcus viridans*, gram-positive and negative bacilli, and staphylococci from the floor of the ulcer, while the emulsion of the wall showed the same streptococcus as the lymph gland, in pure culture.

Cultures from the material aspirated from the floor of the ulcers were made in ten instances, and from the emulsions of the adjacent normal stomach wall in six. Four of the former and three of the latter proved entirely sterile. In five of the former and in one of the latter, yeast cells and sarcinae were found in large or small numbers. These always grew only under strictly aerobic conditions, especially in the upper one-third of the tubes of dextrose agar. A few colonies of staphylococci were found in six of the cultures from material from the floor of the ulcer, and in one of those from emulsions of the stomach wall, and a few colonies of a large, gram-positive, aerobic bacillus in four of the former. Hemolytic and green-producing streptococci developed in small numbers from the material from the floor of the ulcer in three instances, while from the normal stomach wall streptococci did not develop in a single instance.

Altogether, a more or less thorough search for bacteria in stained sections was made in twenty-seven ulcers of the stomach and twenty ulcers of the duodenum. In fifteen of the gastric and ten of the duodenal ulcers, cultures were made; the rest had been preserved in 10 percent formalin for from two to five years, in the museum of the Mayo Clinic. Sections from all ulcers were stained with hematoxylin and eosin. Diplococci or short chains of streptococci were found in the depths of the wall in twenty-one of the ulcers from the stomach (Figs. 2, 3, 4, and 7) and fifteen of those from the duodenum (Figs. 5 and 6). It was found that the best place to search for the streptococci was near areas of leukocytic infiltration in the subperitoneum and along what appeared to be partition membranes or lymph channels. Yeast cells were found in large numbers in three ulcers in the more or less dense connective tissue and in small numbers in nine others of the ulcers of the stomach and in only three of those of the duodenum. In the one ulcer which showed *Staphylococcus aureus* in the cultures, gram-staining cocci were found in the depths of the tissue. No bacteria were found in the adjacent normal mucous membrane in any case, and the floor of the ulcer only occasionally showed bacteria in the sections and, except in the ulcers excised after death, the number of these was small, consisting usually of large saprophytic bacilli, yeast cells, and sarcinae. In two of the lymph glands, a few gram-staining diplococci were found in sections, both having yielded streptococci in cultures (Cases 120 and 126); while in one draining a duodenal ulcer of six months' duration (Case 947), the sec-

tions showed a rather large number of diplococci, and the portion cultured 4,200 colonies of green-producing streptococci in pure culture. Cultures or sections have been made from ulcers or glands draining ulcers, altogether, in fifty-four cases, and gram-staining diplococci or streptococci have been demonstrated in forty-two of these.

A study of the clinical history of the cases of ulcer in conjunction with the sections stained with hematoxylin and eosin and by Gram's method, and the results of the cultures from the lymph glands draining ulcers and of the ulcer wall, show that the number of streptococci is greatest in the relatively acute ulcers and in chronic ulcers in which the clinical history and cellular infiltration indicate a recent lighting up of the infection, and fewest or even absent in the very chronic, markedly indurated ulcers with no clinical microscopic evidence of recent, acute inflammation. Thus, in two cases of gastric ulcer in which yeast colonies were obtained in large numbers, showing their presence, when stained, in the depths of the tissue, in one of which a few streptococci were found and in the other none at all, the ulcers were markedly indurated, sections showed no recent cellular infiltration, and the clinical history no recent increase in symptoms—circumstances suggesting clearly that the ulcers had existed from ten to fifteen years. No such parallelism can be made out in the case of any of the other bacteria.

The results show that the bacterial flora of ulcers excised at operation is very much simpler than that of ulcers excised after death. The number and kinds of bacteria are relatively few, so that, contrary to the view generally held, reliable bacteriologic examination of human gastric and duodenal ulcers can be made if proper methods are employed.

The cultural and other properties of the strains of streptococci isolated, particularly their pathogenicity, are reserved for a later paper. It is sufficient here to state that the strains from twenty-seven cases—all from chronic ulcers—produced small, moist, non-adherent, discrete, grayish-brown or grayish-green colonies on blood (human) agar plates, and produced relatively short chains, and diplococci, and a diffuse turbidity with much acid in dextrose and ascites dextrose broth, and that, when injected into dogs, rabbits, and guinea-pigs, the strains, on isolation, showed a relatively low grade of virulence, but a marked tendency to localize in the mucous membrane of the stomach and duodenum, producing circumscribed areas of infection associated with

hemorrhage and ulceration in a high percentage of the animals inoculated intravenously; that in three cases—all from relatively acute ulcers—streptococci were isolated which produced typical green colonies and usually long chains in dextrose broth, but also showed marked affinity for the mucous membrane of the stomach or duodenum. The streptococci from the ulcers did not show such preference for relatively anaerobic conditions in ascites dextrose broth as had those from rheumatism. The largest number of colonies were found usually in the upper one half of the tube.

The apparently almost constant occurrence of streptococci in the depths of human ulcer, commonly the more numerous the younger the ulcer and the more marked the cellular infiltration, to the total or almost total exclusion of other bacteria, and the fact that when injected into animals such streptococci show a marked tendency to localize in the stomach or duodenum, a property which other strains of bacteria isolated do not possess, taken in conjunction with clinical facts, constitute good evidence that the streptococci are not merely accidental secondary invaders of the tissues, but are commonly the original cause, as well as the important factor in preventing the healing of the ulcer.

EXPLANATION OF PLATES 4 AND 5

PLATE 4

Fig. 1.—Section of chronic, indurated ulcer of the stomach in a man twenty-seven years of age. Note the connective tissue with little or no cellular infiltration and the marked round cell infiltration around blood vessel. $\times 80$.

Fig. 2.—Streptococci and leukocytic infiltration in peritoneal coat in perforating ulcer of stomach in man. $\times 1200$.

Fig. 3.—Streptococci and leukocytic infiltration in peritoneal coat in acute ulcer of stomach in a woman sixty-two years of age. $\times 1200$.

Fig. 4.—Diplococcus in depths of scar tissue far away from ulcerated surface in an indurated ulcer of lesser curvature in a man fifty-six years of age. Ulcer present for five years; no exacerbation of symptoms shortly before operation. $\times 1200$.

PLATE 5

Fig. 5.—Streptococci in depths of chronic (3 yr.) ulcer of duodenum in a woman forty-three years of age. No history of recent acute attack. $\times 1200$.

Fig. 6.—Streptococci between mucous glands at the base of a chronic (4 yr.) ulcer of duodenum with acute symptoms for ten weeks before operation. $\times 1200$.

Fig. 7.—Streptococci and leukocytic infiltration in chronic ulcer with acute exacerbation shortly before operation. $\times 1200$.

Fig. 8.—Diplococci in peritoneal coat of ulcer of the duodenum (see Case 112 in Table 1).

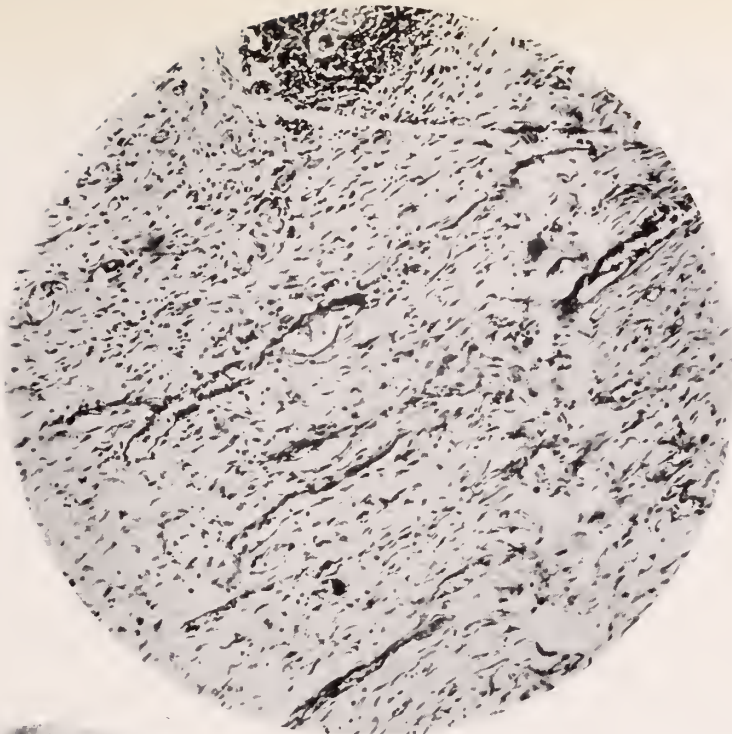


Fig. 1

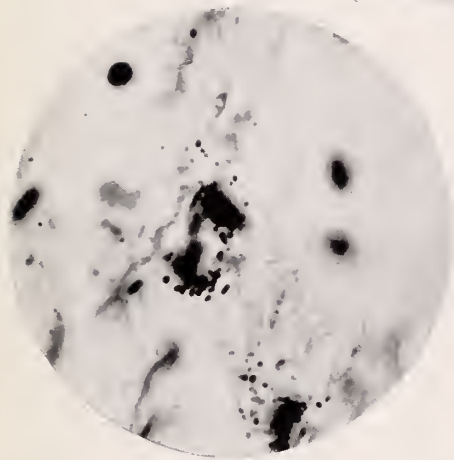


Fig. 2

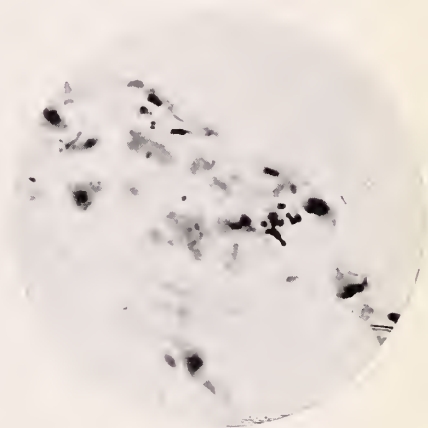


Fig. 3



PLATE 5

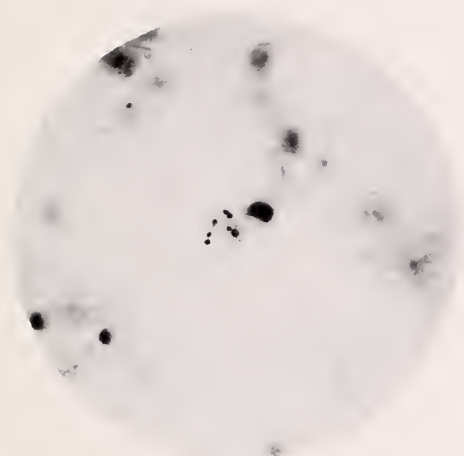


Fig. 5

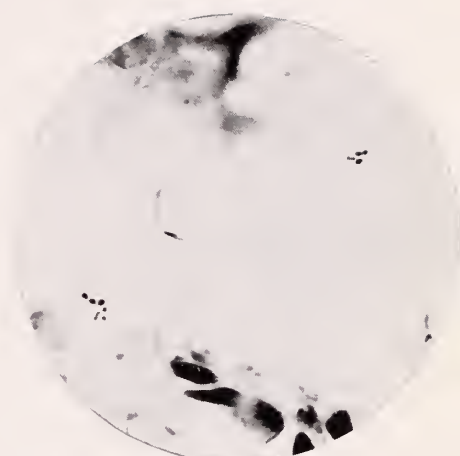


Fig. 6

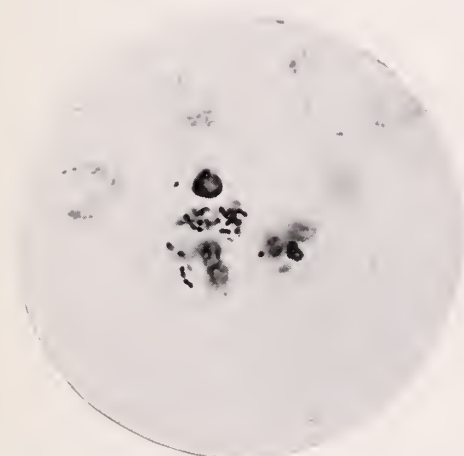


Fig. 7



Fig. 8

FURTHER EXPERIMENTS ON THE VARIABILITY OF THE FERMENTATIVE REACTION OF BACTERIA, ESPECIALLY THE STREPTOCOCCI

WILLIAM C. THRO

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In a preceding paper,¹ after a series of experiments with different colonies derived from one colony, the statement is made that the carbohydrates cannot be used to separate strains of streptococci. In this investigation the media used were agar prepared from sugar-free broth (containing litmus solution) and nutrose water. One criticism of these experiments was that these were poor media on which to grow streptococci. This criticism was anticipated since the use of media containing ascitic fluid or serum, or broth containing meat sugar, was considered inadvisable, the aim being to determine the fermentability of the pure chemicals. On being tested by Benedict's method, samples of three different lots of ascitic fluid were found to contain, respectively, 0.166 gm., 0.125 gm., and 0.147 gm. of glucose per 100 c.c. of fluid.

On reconsidering the matter, it was decided to conduct experiments with ascitic broth with two objects in view: (1) to determine whether a fermentable substance was present in ascitic fluid; (2) to learn whether the colonies of streptococci would also vary in richer media. Before giving the results of the experiments, it seems advisable to give a short statement of the cultural characteristics of the organisms employed.

CULTURAL CHARACTERISTICS OF STREPTOCOCCUS VIRIDANS

The cocci on which these observations have been made were obtained from roots of the teeth and the tooth-sockets in cases of chronic arthritis; from the urine; from blood cultures in endocarditis; from pus in conjunctivitis; from fluid obtained from the ankle joint by puncture; and from the cervix in endometritis. The streptococcus viridans having been obtained so many times from the tonsil, the conclusion is that it is almost universally present.

* Received for publication May 18, 1915.

1. Jour. Inf. Dis., 1914, 15, p. 234.

In Blood Agar.—When tested in human blood agar, some of the strains were distinctly green without any suggestion of hemolysis. Some grew as minute, green colonies with a narrow clear zone. In one case, at the end of twenty-four hours, there was a narrow area of hemolysis that disappeared at the end of forty-eight hours, leaving the green colony. Two strains of the short-chained type, obtained from urine, formed dark brown colonies and a similar result was obtained in several trials, except once when the colonies were green. One black colony, when replated, produced indubitably green colonies. These strains are probably viridans, and the production of brown coloration is yet to be explained. Other strains tried at the same time under identical conditions were green.

In blood plates, if the blood and agar were unevenly distributed, a slight variation in the production of hemolysis and green pigment took place. In areas of the plate where the agar was of greater depth and there was more blood, the colonies were green, while in the areas where the agar was of less thickness, a slight zone of hemolysis was shown. Some of the green colonies being replated, a narrow zone of green hemolysis appeared in the portions of the plate where the agar layer was of slight depth. This variability has been discussed by Anthony,² Ruediger, and Rosenow. All such strains have been grouped with *Streptococcus viridans*.

Morphology.—In broth, if the medium was clear above with a flaky sediment, the chains were usually long, but this did not always hold true. If the broth became uniformly cloudy with granular sediment, the chains were short, but sometimes long ones were produced. Their length was of no value in classification, since some strains formed very long chains; others, chains of medium length; and still others formed chains similar to those usually attributed to *Streptococcus viridans*, i. e. very short ones, up to eight cocci, and even clumps. In one experiment, in which a number of colonies derived from one colony were grown in ascitic broth, two colonies produced long chains and the remainder produced short ones.

On plain agar, the colonies tended to stick to the surface, and in making a vaccine from such the clumps were broken up with difficulty. But in many strains this did not occur.

In litmus milk, some strains quickly coagulated the milk into a firm, white or red mass. Others coagulated the medium slowly. Some strains did not coagulate the medium, but caused it to become faintly or strongly acid. A few did not change it at all. The streptococcus zymogenes, which was green on blood agar, behaved on litmus milk as follows: At the end of twenty-four hours, the milk was partially coagulated, white, with a red stratum at the top. In forty-eight hours it began to peptonize, until it had become a straw-colored fluid with a purplish-red sediment, and finally all the fluid was reddish.

The coagulation of serum inulin as a test to separate pneumococci from streptococci did not always hold true. Of two strains, which were green on blood agar, one, when grown in broth, caused a uniform cloudiness with fine, granular sediment. In the case of the other, the broth was clear above, with flaky sediment. Neither had capsules, but both coagulated and acidified serum inulin. These two would not be classed as pneumococci and yet they fermented inulin.

I have not found as yet any divergence in the bile test.

TABLE 1
SHOWING THE CULTURAL CHARACTERISTICS OF THE ORGANISMS EMPLOYED IN EXPERIMENTS ON THE VARIABILITY OF THE FERMENTATIVE REACTION OF BACTERIA

Organism	Agar	Morphology	Capsules	Blood Agar	Bile + Broth	Litmus Milk	Inulin†	Glucose Serum Agar (Libman)
<i>Pneumococcus</i>	Discrete	Usually diplococci	+	Green	Lysis...	Acid	Fermented	Precipitate rare
<i>Streptococcus mucosus capsulatus</i>	Abundant, confluent, mucoid	Chains of cocci	+ (See Bueger)	Green	Lysis...	Acid	Fermented	Precipitate rare
<i>Streptococcus viridans</i>	Discrete or confluent	Long or short chains, pairs, or groups	±*	Green, brown, or black; slight hemolysis rarely	No lysis	Variable, may coagulate	Not fermented	Precipitate
<i>Streptococcus zymogenes</i>	Discrete or confluent	Chains of cocci or groups	0	Green	No lysis	Peptonized ...	Not fermented	Precipitate
<i>Streptococcus hemolyans</i> (hemolyticus)	Discrete or confluent	Long chains usually	±* (All are gram-positive cocci)	Hemolysis pronounced	No lysis	Variable	Not fermented	Precipitate

* Occasional cocci show a narrow capsule, not comparable to that found on pneumococci, however.

† Not infallible.

Several experiments were conducted to determine whether there is enough sugar in the ascitic fluid to produce an appreciable amount of acid. Thirteen colonies derived from one colony of *Streptococcus viridans* were placed in sugar-free broth, three parts, ascitic fluid, one part, plus litmus solution. There was slight variation in the amount of acid. Some of the same medium was inoculated with *Bacillus coli* to determine whether there was enough dextrose present to change the reaction. The control showed 0.3 c.c. n/10 NaOH, while the inoculated medium varied from 0.55 to 0.6 c.c. This experiment was repeated by the inoculation of twelve tubes of ascitic broth with *Bacillus coli*. Two controls titrated alike, that is, 0.8 c.c. of decinormal soda hydroxid. The inoculated tubes varied from 0.8 to 1.2 c.c. These experiments seem to prove the presence of a fermentable substance in ascitic fluid, determined to be glucose, as previously stated.

In the following experiments, the medium used was sugar-free broth (made from fresh meat), three parts, and sterilized ascitic fluid, one part. When carbohydrate was used, it was added, in the proportion of 1 percent, after it had been separately sterilized for twenty minutes in the Arnold sterilizer. In the titration experiments, 10 c.c. of culture media were used. The indicator, phenolphthalein, was titrated against decinormal NaOH. Of course, the cultures were examined for contaminations before titration.

Twenty colonies isolated from one original colony of *Streptococcus viridans* 57145, isolated from a tonsil, were placed in the medium described, containing mannite and litmus solution. In most of the tubes, the reaction was faintly acid; in four, it was very faintly acid; in one, it was very acid; and in four, the reaction was neutral.

Eight original colonies of *Streptococcus viridans* from a tonsil were planted in salicin ascitic broth. On titration there was a variation from 0.6 c.c. to 3.0 c.c. (control, 0.2 c.c.).

Fifteen colonies derived from one of these eight original colonies were grown in salicin ascitic broth. The titration showed a variation from 1.6 c.c. to 3.2 c.c. (control, 0.2 c.c.).

Twenty-four original colonies of *Streptococcus hemolysans* (hemolyticus) from an abscess were grown in raffinose ascitic broth. On titration the variation was not great, being from 0.3 c.c. to 0.7 c.c.

Twelve colonies from one of these colonies, grown in the same media, varied from 0.6 c.c. to 1.2 c.c.

Twenty-three original colonies of *Streptococcus viridans* isolated from a tonsil were planted in raffinose ascitic broth. On titration there was a variation from 0.2 c.c. to 2.3 c.c.

Four colonies of *Streptococcus viridans* obtained from one of the twenty-three original colonies were planted in raffinose ascitic broth. On titration there was a variation from 0.6 c.c. to 2.9 c.c. (control of ascitic broth, 0.65 c.c.).

Five original colonies of *Streptococcus viridans* from a tonsil were planted in ascitic sugar-free broth. On titration they were found to be nearly alike, 0.5 c.c. to 0.75 c.c. (control, 0.65 c.c.). In this experiment, there was no variation and not much acid produced.

Fifteen original colonies of *Streptococcus viridans* from a tonsil were grown forty-eight hours in 1 percent salicin ascitic broth. On titration there were variations from 0.3 c.c. to 5.2 c.c. In the tubes in which there was the best growth, the largest amount of acid was produced.

Nineteen colonies (derived from one colony) of *Streptococcus viridans* from a tonsil were grown in salicin. On titration there was a variation from 0.55 c.c. to 2.6 c.c. Three colonies produced long chains; the remainder, short chains.

Sixteen colonies of this *streptococcus viridans* derived from one colony, were grown in raffinose; the variation in this experiment was not large, that is, from 0.6 c.c. to 0.95 c.c.

Seventeen colonies derived from one colony of a *streptococcus* that was neither *viridans* nor *hemolysans* (*hemolyticus*), were grown on raffinose and showed variations from 0.6 c.c. to 1.0 c.c.

Twelve of the same colonies were grown in salicin, and showed a variation from 4.0 c.c. to 4.6 c.c.

In the last two experiments, it is seen that the variations were not great. In these experiments the *streptococcus* grew very luxuriantly.

Twenty-one colonies derived from one colony of *Streptococcus viridans* 59288, were grown in salicin ascitic broth. In every tube, there was a luxuriant growth of short-chained *streptococci*, and there

was only a slight variation on titration, that is, from 4.0 c.c. to 4.5 c.c. of $n/10$ NaOH (control, 0.3 c.c.).

Sixteen colonies derived from one colony of *Streptococcus viridans* 59856 were planted in salicin ascitic broth. Since there were no signs of growth in some tubes at the end of twenty-four hours, they were re-inoculated with large amounts of bacteria. On titration it was found that the amount of acid varied exactly with the amount of growth. The titration showed variations from 0.25 c.c. to 3.3 c.c. In the tubes in which no acid was produced, there was no growth. The cultures from which the latter were inoculated were tested on North's medium and were found to be alive. Evidently, then, some colonies seem disinclined to grow even in ascitic broth.

Twelve colonies derived from one of these luxuriantly growing colonies were planted in mannite ascitic broth plus litmus. Four colonies showed a faint acid reaction; the others were neutral. In this instance the variation was slight.

Twelve colonies derived from one colony of *Streptococcus viridans* 59856 were planted in salicin ascitic broth. Here, again, the amount of acidity agreed with the luxuriance of the growth, the amount of acidity varying from 0.6 c.c. to 3.7 c.c.

Some of these colonies that showed a great variation were planted in a new lot of medium. There was luxuriant growth in each tube with the result that the titrations were nearly alike.

Twelve colonies derived from one colony of *Streptococcus viridans* 59768 were grown in salicin ascitic broth and varied from 0.6 c.c. to 4.1 c.c. In the tubes in which the broth titrated 0.6 c.c., there was no growth.

Nine original colonies of *Bacillus rhinoscleromatis* were grown in stabs on ascitic agar plus saccharose. At the end of three days, three were neutral, six were acid, and two had from 6 to 7 gas bubbles in the agar. By the sixth day all were acid. On the second transplant, by the fourth day, all were acid and two still showed gas bubbles. The nine colonies were placed on lactose, and at the end of the ninth day, two tubes were neutral and seven were very faintly acid.

CONCLUSIONS

One factor determining the variations in the amount of acidity is certainly the variability of the luxuriance of growth of micro-organisms.

It seems inadvisable, in any exact experiments on the variability of micro-organisms on the carbohydrates, to use ascitic fluid or broth that has not had the meat sugar removed from it.

Undoubtedly the streptococci will grow better on broth from which the meat sugar has not been exhausted, and in ascitic broth, but the results with such media and carbohydrates are questionable, since glucose in the meat extract and ascitic fluid is readily fermented by all streptococci.

AN INFECTION WITH THE PARATYPHOID BACILLUS (*B. PARATYPHOSUS* B) *

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The case here reported has afforded a somewhat unusual opportunity for a complete clinical and bacteriologic study of an infection with the paratyphoid bacillus. An acute infection led to the condition of chronic paratyphoid carrier, associated with recurrent attacks of cholecystitis that finally necessitated drainage of the gall-bladder, with subsequent disappearance of paratyphoid bacilli from the stools. The report follows:

Miss Y. Z., 23 years old. Family history shows nothing of note; previous illnesses were measles, chicken pox, pertussis, in childhood. She had never had typhoid fever. In 1906, after repeated attacks of pain in the abdomen, the appendix was removed with relief of symptoms. In 1911, she had an attack of pain in the right ankle which was diagnosed "rheumatism." Otherwise health had been good and patient had been able to attend to her duties as a nurse. On October 11, 1912, the patient entered the Presbyterian Hospital, in the service of Dr. J. B. Herrick, suffering from severe, spasmodic pain in the right hypochondrium, accompanied by nausea, vomiting, and fever. This was the fourth of a series of attacks during the previous five weeks, all of which were of similar character, sudden in onset and usually associated with fever, varying only in severity. Periods of apparently good health intervened. There was no previous trouble referable to the stomach, and all attacks came on without any demonstrable relation to the taking of food. Since the last attack two weeks before, the patient thought that possibly she had had fever; she had felt ill, tired, and had had a poor appetite.

She was a well-nourished young woman, apathetic, except when roused by a paroxysm of pain in the abdomen. The region of hypochondrium was extremely tender, with a less degree of tenderness over the remaining upper abdomen. There was no general rigidity. Temperature was 101 F.; pulse 108; respirations 24. A complete physical examination revealed no other abnormalities of note.

Blood: Leukocytes 14,000; hemoglobin 85 percent (Dare).

Urine: Clear, amber, acid, containing no albumin, sugar, casts, or pus.

Stool: Yellowish brown, showing no blood or other abnormalities. Five subsequent examinations of the stools showed traces of blood by the Weber test (menstruation in progress).

Oct. 13.—During previous two days, temperature remained between 100 and 102 F. Repeated vomiting of greenish fluid containing faint traces of blood.

Blood: Leukocytes 10,700; hemoglobin 90 percent (Dare).

Oct. 15.—Temperature rose steadily and for twenty-four hours varied between 103 and 105 F. Pulse 110-115. Blood culture made.

Blood: Leukocytes 8,200. A count of 200 cells gave 87.5 percent polymorphonuclears; 12.5 percent mononuclears.

Oct. 16.—Leukocytes 6,200; polymorphonuclears 80 percent; mononuclears 20 percent. Urine: Cloudy, 1.017, albumin trace, no sugar, acetone present, no diacetic, many hyaline and granular casts, a few leukocytes and red cells.

Stool: Brown, liquid, Weber positive, no amebae.

Oct. 17.—Leukocytes 8,300. Polymorphonuclears 87.5 percent; mononuclears 12.5 percent.

Urine: Albumin increased; large numbers of hyaline, granular, and epithelial casts together with red cells and leukocytes.

Slight abdominal tenderness still present. The appearance is more suggestive of typhoid fever. Lips, dry; a slight sore throat; pulse, dicrotic. The temperature remained high, varying between 103 and 105 F. The blood culture in broth showed a motile, gram-negative bacillus. Agglutination with *B. typhosus* negative at 1:20 and 1:40, in one hour.

Oct. 18.—Suspicious rose spots over abdomen.

Oct. 19.—General condition improved, but there were marked drowsiness and apathy.

Urine: 1.012, albumin trace, casts decreased in number.

Oct. 20.—Patient more somnolent. Vomiting ceased. Tongue heavily coated in center with margin red. Lips dry. On abdomen six to eight small, red, hyperemic macules. Spleen not palpable. Agglutination with *B. typhosus* one hour 1:40 negative; with *B. paratyphosus B*, positive.

During the succeeding ten days the patient was at times irrational. Some tenderness over the region of the gall-bladder.

Nov. 2.—Temperature falling by lysis. High point on this date 99.2 F. Appetite good; no nausea. Abdomen soft, but some tenderness at the right costal margin.

Nov. 4.—Patient complained of sudden, severe pain in the right lower quadrant. When seen a few minutes later, she was tossing restlessly in bed. Pulse 118. Temperature 98.6 F. Leukocytes 6,400. After a few minutes the pain lessened, but recurred for brief periods at intervals throughout the day. No signs of local inflammation. Temperature remained normal, and leukocytes were not increased. The patient was irrational at times.

Nov. 8.—A slow improvement in mental condition. Urination involuntary for the past five days. An erythema with severe itching present over arms, legs, and abdomen for the past four days.

Nov. 11.—Blood: Erythrocytes 4,200,000; leukocytes 9,000; hemoglobin 72 percent (Dare).

Nov. 26.—Convalescence rapid. Some tachycardia on exertion, with a coarse tremor of the hands, and a slight increase in size of the thyroid. General condition good.

Blood: Erythrocytes 5,100,000; leukocytes 7,100; hemoglobin 85 percent (Dare).

May 22, 1913.—The patient had been in apparently good health, with the exception of an occasional, slight pain below the right costal margin. At 4 a. m. she was awakened by a sharp, cramp-like pain in the epigastrium, which became very severe; some nausea. After one-half hour the pain became less. A chill lasting several minutes followed. Temperature normal. This attack was very similar to those occurring eight months previously and did not follow any indiscretion in diet. No jaundice. Leukocytes 11,000. Agglutination with *B. paratyphosus B.* one hour 1:50 positive; 1:100, negative.

Jan. 13, 1914.—Following recurrences of the attacks of pain in the right hypochondrium in July, 1913, and again in January, 1914, drainage of the gall-bladder was performed by Dr. Dean D. Lewis. The wall of the gall-bladder was much thickened and contained dark bile. There were no calculi in the gall-bladder or in the ducts. The patient made a good recovery from the operation, and has had no return of the symptoms.

SUMMARY

The onset of illness presented several puzzling features which left the diagnosis in doubt for the first few days. The earlier attacks of epigastric pain seemed clearly referable to the gall-bladder, but the severe attack followed by fever, leukocytosis, and prostration raised the question of an acute condition calling for surgical treatment, such as suppurative cholecystitis or perforation of a duodenal ulcer. After the subsidence of the acute upper abdominal symptoms, the appearance of albumin, red cells, leukocytes, and numerous casts, associated with continued fever, recalled the occasional onset of typhoid fever with acute renal symptoms. *B. paratyphosus B.* was isolated from the blood drawn on the fifth day of acute illness, and agglutination with a laboratory strain of *B. paratyphosus B.* was obtained on the tenth day.

The subsequent course was that of a severe typhoid, with continued fever ranging from 103 F. to 105 F., falling gradually in the fourth week. The pulse was more rapid (110-120) than usual in typhoid during the first weeks of the illness. In the fifth week, a pronounced psychosis developed, and lasted several days with delusions, more marked toward night. At the same time, erythematous, itching lesions appeared over arms, abdomen, and legs, persisting for a week, and during this period there was a recurrence of epigastric pain similar to attacks noted at the onset of the illness, but without leukocytosis or fever. During the subsequent weeks of convalescence, the pulse was irritable, rising on slight exertion, and a slight, temporary enlargement of the thyroid was noted. The later convalescence was uneventful.

A careful search was made for the source of infection. The patient had not received prophylactic typhoid inoculation. She had assisted in tubbing a man suffering from typhoid fever during the weeks previous to her illness. The blood of this man had given prompt agglutination with *B. typhosus*. A detailed examination of persons handling the food supply in the hospital where she was employed as nurse, made somewhat later for another purpose, showed no evidence of infection by *B. paratyphosus B*. It is possible that infection may have occurred through an undetected paratyphoid carrier, among the ward patients, or from a source outside the hospital, but we could obtain no positive evidence on this point.

These investigations were made in the hope of throwing some light on the question of whether we were dealing with an acute, recent infection with *B. paratyphosus B* in a person already suffering from recurrent cholecystitis over a period of five weeks, or whether the cholecystitis was an early manifestation of the infection which later became general. The close association of the two periods of illness, and the later development of the carrier state, with the demonstration of *B. paratyphosus B* in the gall-bladder are suggestive, but the evidence hardly warrants a definite conclusion as to the relation of the two illnesses.

BACTERIAL EXAMINATION *

A bacillus of the *B. paratyphosus* type was isolated uniformly from this patient. The bacillus is gram-negative, actively motile, does not liquefy gelatin, produces gas in dextrose broth, and forms alkali in litmus milk. It produces no indol. Acid and gas are formed in the following carbohydrate media: dulcitol, mannitol, galactose, sorbitol, arabinose, levulose, maltose, mannitol, xylose, and isodulcitol, but not in lactose, saccharose, salicin, raffinose, inulin, dextrin, glycerin, erythritol, and adonitol.

The serum of a rabbit immunized with a typical culture of *B. paratyphosus B* (from the collection of the Museum of Natural History, New York, originally obtained from the Rockefeller Institute) agglutinated this organism (titer 1:2000) as completely as the homologous strain. Controls with *Bacillus typhosus* serum and *Bacillus enteritidis* (Gärtner) serum were negative.

* A portion of the expense for this examination was borne by the Serum Division of the Memorial Institute for Infectious Diseases.

Strains with all the characteristics mentioned were obtained in this case as follows:

Source	Date	Bacillus Paratyphosus B Isolated
Blood	10-17-1912	(Pure culture)
Feces	10-22	—
Feces	11- 5	(Mostly <i>B. coli</i> , very few <i>B. paratyphosus B</i>)
Feces	11-19	(About $\frac{1}{3}$ <i>B. paratyphosus</i>)
Feces	11-26	—
Feces	11-24-1913	(About $\frac{1}{3}$ <i>B. paratyphosus</i>)
Urine	11-24	—
Bile (at operation)	1-13-1914	(Pure culture)
Feces	5-25	—
Feces	6- 1	—
Feces	6-17	—
Feces	11-12	—
Feces	11-21	—
Feces	11-25	—
Feces	12- 3	—
Feces	12- 9	—
Feces	1-27-1915	—
Feces	2- 3	—
Feces	2-11	—
Feces	5- 3	—
Feces*	6-15	—

* Obtained after administration of a purgative.

On May 3, 1915, the agglutinative power of the blood was found to be positive for *B. paratyphosus B* in 1:200 dilution and negative for *B. typhosus* at 1:50.

In each case where successful isolation is noted, several strains of the organism were obtained, and all these resembled one another in every respect. The fermentation reactions of all the strains were identical also with those of the *B. paratyphosus B* cultures (Rockefeller Institute) used for immunizing the rabbit, and with the other laboratory stock strains of the University of Chicago collection labeled *B. paratyphosus B*.

The apparent disappearance of the paratyphoid bacillus from the stools after drainage of the gall-bladder is of particular interest as bearing on the treatment of chronic typhoid and paratyphoid carriers. Operative procedure has been advocated especially by Dehler,¹ who found in his observation of four typhoid carriers that cholecystectomy was followed by practical disappearance of typhoid bacilli from the stools. In one of the cases reported by Dehler, thirty-seven examinations of the feces during the two years before operation resulted in finding *B. typhosus* abundantly every time, while in the first six months after operation seventy-six examinations showed the presence of *B. typhosus* but once and then in small numbers. Similar observations

1. München. med. Wehnschr., 1907, 54, pp. 779-2134; *ibid.*, 1912, 59, p. 857.

have been made by Blumenthal and Grimme,² Daeschler,³ Leary,⁴ O. Mayer,⁵ and others, but in some of these cases, examination of the feces was continued for only a few weeks after operation. It is evident from other observations that drainage or extirpation of the gall-bladder is not always effective in causing the permanent disappearance of typhoid bacilli from the stools. In the instance described by Loele,⁶ the typhoid bacilli found in the feces apparently did not come from the gall-bladder but from the bile duct or diverticula of the upper intestine, as indicated by the autopsy. Fromme⁷ found typhoid bacilli in the liver five days after extirpation of the gall-bladder, and in another case, after removal of an infected gall-bladder, isolated them for some days from the drainage tube in almost pure culture, and concludes therefore that the gall-bladder is not the only seat of vegetation. O. Mayer,⁸ altho noting the disappearance of typhoid bacilli from the stools after extirpation of the gall-bladder, does not believe that this disappearance is lasting in all cases and quotes the expression of Messerschmidt to the effect that after a time typhoid bacilli are again found.

Gall-bladder infections with *B. paratyphosus B* have been recorded by Forster and Kayser,⁹ Lorey,¹⁰ Eckersdorff,¹¹ Evers and Mühlens,¹² and Pribram.¹³ In the case described by Forster and Kayser, *B. paratyphosus* was isolated from the gall-bladder at autopsy; in Eckersdorff's case, the existence of cholecystitis was determined on clinical grounds and a paratyphoid bacillus—agglutinating like the Schottmüller strain—was isolated from the feces. Neither case was submitted to operation.

Lorey's case was one of operation (cholecystectomy) after typhoid (?) of two years' standing, following which "several" examinations failed to show paratyphoid bacilli in the feces, altho these organisms were present before operation. The case reported by Evers and Mühlens was more carefully studied; during the six weeks following operation for gallstones (cholecystotomy), *B. paratyphosus B* was iso-

2. München. med. Wchnschr., 1908, 55, p. 16; Deutsch. Arch. f. klin. Med., 1906, 88, p. 509.

3. Centralbl. f. Bakteriöl., I, O., 1912, 52, p. 283.

4. Jour. Am. Med. Assn., 1913, 60, p. 1293.

5. München. med. Wchnschr., 1914, 61, p. 1116.

6. Deutsch. med. Wchnschr., 1909, 35, p. 1429.

7. Deutsch. Ztschr. f. Chir., 1910, 107, p. 578.

8. München. med. Wchnschr., 1914, 61, p. 1116.

9. Ibid., 1905, 52, p. 1473.

10. Ibid., 1908, 55, p. 15.

11. Arb. a. d. k. Inst. f. exper. Therap. zu Frankfurt, 1908, 4, p. 61.

12. Deutsch. militärärzt. Ztschr., 1909, 38, p. 366.

13. Wien. klin. Wchnschr., 1912, 25, p. 1344.

lated from the feces in four out of eight examinations, and there was no evidence that the operation was successful in removing the patient from the ranks of carriers. Pribram describes an especially interesting case in which a patient was found to be excreting paratyphoid bacilli three years after removal of the gall-bladder.

The case here described is noteworthy for the occurrence of general paratyphoid infection in a person who for a month previously had suffered from recurrent attacks of cholecystitis; for the recurrence of the cholecystitis after convalescence, associated with persistence of paratyphoid bacilli in the stools; for the isolation of the bacilli from the bile at operation; and for complete cessation of gall-bladder symptoms accompanied by disappearance of paratyphoid bacilli from the stools during more than one year following operation (cholecystotomy). During the latter part of the acute illness, no clinical difference could be noted between the infection and a rather severe attack of typhoid fever, and a correct diagnosis could be made only as the result of bacteriologic examination. At no time was there any evidence that this carrier was giving rise to contact cases.

INHIBITORY ACTION OF HETEROLOGOUS PROTEIN MIXTURES ON ANAPHYLAXIS*

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In studying the absorption of serum from the subcutaneous tissues, a delicate method was needed to detect small amounts of foreign protein in the circulation. Since from many investigations (Rosenau and Anderson,¹ and Wells,²) we know that extremely small amounts of protein will sensitize guinea-pigs, the anaphylaxis reaction was indicated as an excellent means to measure the amount, rate, and channel of absorption of serum from the subcutaneous tissues. This method has been used by several investigators to show the presence of foreign protein in the circulation, most recently by Eleanor Van Ness Van Alstyne,³ who asserts that she has thus shown whole protein to be absorbed through the gastro-intestinal tract.

Our method of procedure was to anesthetize a medium-sized dog and insert cannulae into the thoracic duct and a small artery in the neck. Varying amounts of horse serum were injected under the skin of the abdomen, and at stated intervals of time samples of 5 to 10 c.c. lymph and blood were taken. From each sample 1.5 to 2 c.c. of serum were injected intraperitoneally into guinea-pigs. After fourteen days, these were tested for sensitization by injecting intraperitoneally 0.5 to 1 c.c. horse serum.

Negative results were always obtained, even under conditions where we had reason to believe that large enough amounts of horse serum to sensitize guinea-pigs ought to have been contained in 2 c.c. of serum. Under similar conditions, colloid dyes of large, molecular dimension appeared promptly in the blood and lymph. In an attempt to obtain positive results, 50 c.c. of horse serum were forced under the skin of a dog with compressed air, in much the same way that anatomists inject the subcutaneous lymphatics with mercury; but even after this drastic procedure, the dog's blood did not sensitize guinea-pigs.

* Received for publication May 26, 1915.

1. Hygienic Laboratory Bull. 29, 1906.

2. Jour. Infect. Dis., 1911, 9, p. 449.

3. Arch. Int. Med., 1913, 12, p. 372.

to horse serum. Sensitized guinea-pigs, when injected subcutaneously with large amounts of antigen in this way, die promptly as with an intravenous injection.

In order to find wherein the difficulty lay, the following experiment was made to find whether a mixture of dog serum with a definite amount of horse serum could sensitive guinea-pigs to horse serum.

On October 7, 1914, a dog under ether was bled aseptically from the carotid artery. After coagulation, the blood was centrifugated and the clear serum drawn off. Into each of six test tubes were immediately put 2 c.c. of the serum. To these test tubes were added respectively 0.1, 0.01, 0.001, 0.0001, and 0.00001 c.c. horse serum. Each amount of horse serum was diluted with NaCl solution so that it was contained in 1 c.c. The tubes were put in the ice-box over night. The next morning they were heated to 56 C. for one-half hour. Fresh dog serum is toxic, and to avoid killing the guinea-pigs, it is usually either heated or injected at intervals. Twenty-four hours after the blood was withdrawn, the contents of each tube were injected intraperitoneally into a guinea-pig. As controls, two guinea-pigs were given 0.1 and 0.01 c.c. horse serum diluted to 3 c.c.

On October 26, all guinea-pigs received intraperitoneally, 1 c.c. horse serum diluted to 2 c.c. The results are seen in Table 1.

TABLE 1
THE EFFECT OF DOG SERUM ON THE ACTIVE SENSITIZATION OF GUINEA-PIGS WITH
HORSE SERUM

Guinea-Pigs	First Injection	Reaction After Second Injection (Intraperitoneal) of 1 c.c. Horse Serum Diluted One-Half
1	0.1 c.c. horse serum 2 c.c. dog serum	Slight ruffling of coat
2	0.01 c.c. horse serum 2 c.c. dog serum	Questionable
3	0.001 c.c. horse serum 2 c.c. dog serum	None
4	0.0001 c.c. horse serum 2 c.c. dog serum	None
5	0.00001 c.c. horse serum 2 c.c. dog serum	None
6	0.1 c.c. horse serum 2 c.c. NaCl solution	Dead in 35 min.
7	0.01 c.c. horse serum 2 c.c. NaCl solution	Marked within 10 min.; prostrated in 25 min. Symptoms lasted over 6 hours. Recovered

The results show that anaphylactic sensitization has been almost completely suppressed as the result of mixing the sensitizing dose with dog serum. To make sure, the experiment was repeated several times, using more complete controls.

On November 2, 1.5 c.c. were measured from nine dilutions of horse serum that contained in each cubic centimeter, respectively, 0.5, 0.1, 0.01, 0.002, 0.001, 0.0001, 0.00005, 0.00002 and 0.00001 c.c. of horse serum. This amount was put into test tubes containing 3 c.c. of freshly drawn dog serum. Similar amounts were measured into test tubes containing 3 c.c. of NaCl solution. All tubes were well shaken and kept at room temperature twenty-one hours, then heated to 56 C. for one-half hour. When cooled to body temperature, 3 c.c. from each tube were injected intraperitoneally into a guinea-pig. Thus each animal received 2 c.c. of dog serum or NaCl solution plus the amount of horse serum contained in 1 c.c. of one of the dilutions.

On November 10, each animal received 1 c.c. of horse serum diluted to 2 c.c. The results are seen in Table 2.

TABLE 2
THE EFFECT OF DOG SERUM ON THE ACTIVE SENSITIZATION OF GUINEA-PIGS WITH
HORSE SERUM

Guinea-Pigs	First Injection	Reaction After Second Injection (Intraperitoneal) of 1 c.c. Horse Serum Diluted One-Half
1	0.1 c.c. horse serum 2 c.c. dog serum	None
2	0.01 c.c. horse serum 2 c.c. dog serum	Scratches nose occasionally
3	0.002 c.c. horse serum 2 c.c. dog serum	None
4	0.001 c.c. horse serum 2 c.c. dog serum	None
5	0.0001 c.c. horse serum 2 c.c. dog serum	None
6	0.00005 c.c. horse serum 2 c.c. dog serum	None
7	0.00002 c.c. horse serum 2 c.c. dog serum	None
8	0.00001 c.c. horse serum 2 c.c. dog serum	None
9	0.01 c.c. horse serum 2 c.c. NaCl solution	Marked; prostrated. Died during night
10	0.002 c.c. horse serum 2 c.c. NaCl solution	Marked; prostrated. Recovered
11	0.001 c.c. horse serum 2 c.c. NaCl solution	Died during night
12	0.0001 c.c. horse serum 2 c.c. NaCl solution	Severe; prostrated. Recovered
13	0.00005 c.c. horse serum 2 c.c. NaCl solution	Strongly positive
14	0.00002 c.c. horse serum 2 c.c. NaCl solution	Positive
15	0.00001 c.c. horse serum 2 c.c. NaCl solution	Positive

On November 23, 9 c.c. of the serum from freshly drawn dog blood were mixed thoroughly with 1 c.c. of horse serum. The mixtures were put into the incubator at 38 C. for two hours. A few drops of CHCl_3 were then added and the whole kept at room temperature until the next morning. The following dilutions of the mixture were then made: 1:10, 1:50, 1:100, 1:1000, 1:10000, 1:20000. One cubic centimeter of these dilutions contained therefore, 0.01, 0.002, 0.001, 0.0001, 0.00005, 0.00002, and 0.00001 c.c. of horse serum and nine times as much dog serum. A similar series was made using NaCl solution instead of dog serum. One cubic centimeter of each of the dilutions was injected intraperitoneally into a guinea-pig.

TABLE 3

THE EFFECT OF DOG SERUM ON THE ACTIVE SENSITIZATION OF GUINEA-PIGS WITH HORSE SERUM

Guinea-Pigs	First Injection	Reaction After Second Injection (Intraperitoneal) of 1 c.c. Horse Serum Diluted One-Half
1	0.01 c.c. horse serum in dog serum	None
2	0.002 c.c. horse serum in dog serum	None
3	0.001 c.c. horse serum in dog serum	None
4	0.0001 c.c. horse serum in dog serum	None
5	0.00005 c.c. horse serum in dog serum	None
6	0.00002 c.c. horse serum in dog serum	None
7	0.00001 c.c. horse serum in dog serum	None
8	0.01 c.c. horse serum in NaCl solution	Very severe; prostrated in 1 hour Recovered
9	0.002 c.c. horse serum in NaCl solution	Very severe; prostrated in 1 hour Recovered
10	0.001 c.c. horse serum in NaCl solution	Very severe; prostrated. Recovered
11	0.0001 c.c. horse serum in NaCl solution	Marked. Recovered
12	0.00005 c.c. horse serum in NaCl solution	None
13	0.00002 c.c. horse serum in NaCl solution	None
14	0.00001 c.c. horse serum in NaCl solution	None

On December 3, all animals received intraperitoneally 1 c.c. of horse serum. The results are shown in Table 3.

On December 21, to 9 c.c. of fresh dog serum and to 9 c.c. of NaCl solution, 1 c.c. of horse serum was added and well mixed. The mixtures were put

into the incubator two hours, and into the ice-box over night (twenty hours). Dilutions of each of the mixtures were made so that in 5 c.c. would be contained 0.5 c.c., 0.25 c.c., 0.1 c.c., 0.02 c.c., 0.01 c.c., and 0.002 c.c. horse serum. Five cubic centimeters of each of these dilutions were injected into a guinea-pig. The dilutions that contained 0.5 c.c. horse serum were given in three injections at intervals of one-half hour (in order to prevent the toxic action of fresh dog serum) and those that contained 0.25 c.c. were given in two injections at an interval of one-half hour. The other dilutions were given in one injection.

On January 14, all animals were given, intraperitoneally, 1 c.c. horse serum diluted to 2 c.c. The results are seen in Table 4.

TABLE 4
THE EFFECT OF DOG SERUM ON THE ACTIVE SENSITIZATION OF GUINEA-PIGS WITH HORSE SERUM

Guinea-Pigs	First Injection	Reaction After Second Injection (Intraperitoneal) of 1 c.c. Horse Serum Diluted One-Half
1	0.5 c.c. horse serum in dog serum	Very slight
2	0.25 c.c. horse serum in dog serum	Slight restlessness
3	0.1 c.c. horse serum in dog serum	Restless
4	0.02 c.c. horse serum in dog serum	None
5	0.01 c.c. horse serum in dog serum	None
6	0.002 c.c. horse serum in dog serum	None
7	0.5 c.c. horse serum in NaCl solution	Immediate; convulsions; died during night
8	0.25 c.c. horse serum in NaCl solution	Severe. Recovery
9	0.1 c.c. horse serum in NaCl solution	Severe. Recovery
10	0.02 c.c. horse serum in NaCl solution	Dead in 30 minutes
11	0.01 c.c. horse serum in NaCl solution	Very severe; prostrated. Recovered
12	0.002 c.c. horse serum in NaCl solution	Very severe. Recovery

It will be seen that the results are consistent. While the controls showed the usual symptoms of anaphylaxis, the guinea-pigs that received dog serum showed few or no symptoms. The difference was shown very strikingly when, after the toxogenic dose, the cage containing one series was placed beside the one containing the other series.

In seeking for an explanation of this phenomenon, it was at first thought that the dog serum had a specific action on the horse serum of an antibody-antigen nature which attenuated its ability to act as an antigen, because in one instance there was a slight precipitate seen in three of the dog-serum horse-serum mixtures (Table 3, Guinea-pigs 1, 2, and 3). In order to find whether this property was confined to

TABLE 5

THE EFFECT OF DOG SERUM AND EGG-WHITE ON THE ACTIVE SENSITIZATION OF GUINEA-PIGS WITH HORSE SERUM

Guinea-Pigs	First Injection	Second Injection (Intraperitoneal)	Reaction
1	$\frac{1}{2}$ c.c. horse serum 2 c.c. dog serum	0.5 c.c. horse serum diluted one-half	Indefinite
2	$\frac{1}{6}$ c.c. horse serum 2 c.c. dog serum	0.5 c.c. horse serum diluted one-half	Slight
3	$\frac{1}{15}$ c.c. horse serum 2 c.c. dog serum	0.5 c.c. horse serum diluted one-half	None
4	$\frac{1}{75}$ c.c. horse serum 2 c.c. dog serum	1 c.c. horse serum diluted one-half	None
5	$\frac{1}{150}$ c.c. horse serum 2 c.c. dog serum	1 c.c. horse serum diluted one-half	None
6	$\frac{1}{750}$ c.c. horse serum 2 c.c. dog serum	1 c.c. horse serum diluted one-half	None
7	$\frac{1}{2}$ c.c. horse serum 2 c.c. egg-white solution	0.5 c.c. horse serum diluted one-half	None
8	$\frac{1}{6}$ c.c. horse serum 2 c.c. egg-white solution	0.5 c.c. horse serum diluted one-half	None
9	$\frac{1}{15}$ c.c. horse serum 2 c.c. egg-white solution	0.5 c.c. horse serum diluted one-half	None
10	$\frac{1}{75}$ c.c. horse serum 2 c.c. egg-white solution	1 c.c. horse serum diluted one-half	None
11	$\frac{1}{150}$ c.c. horse serum 2 c.c. egg-white solution	1 c.c. horse serum diluted one-half	None
12	$\frac{1}{750}$ c.c. horse serum 2 c.c. egg-white solution	1 c.c. horse serum diluted one-half	None
13	$\frac{1}{3}$ c.c. horse serum 2 c.c. NaCl solution	0.5 c.c. horse serum diluted one-half	Very severe; prostrated. Recovery.
14	$\frac{1}{6}$ c.c. horse serum 2 c.c. NaCl solution	0.5 c.c. horse serum diluted one-half	Severe; prostrated. Re- covery
15	$\frac{1}{15}$ c.c. horse serum 2 c.c. NaCl solution	0.5 c.c. horse serum diluted one-half	Very marked
16	$\frac{1}{75}$ c.c. horse serum 2 c.c. NaCl solution	1 c.c. horse serum diluted one-half	Dead in 25 minutes
17	$\frac{1}{150}$ c.c. horse serum 2 c.c. NaCl solution	1 c.c. horse serum diluted one-half	Dead in 60 minutes
18	$\frac{1}{750}$ c.c. horse serum 2 c.c. NaCl solution	1 c.c. horse serum diluted one-half	Very marked

dog serum, the experiment was repeated using human serum and cat serum. Results followed identical in nature with those obtained with dog serum.

As the sera of man, dog, and cat are very toxic for guinea-pigs, another possibility was conceived, namely, that they acted deleteriously

TABLE 6

SENSITIZATION OF GUINEA-PIGS WITH A LARGE AMOUNT OF AN ANTIGEN INJECTED WITH A SMALLER AMOUNT OF ANOTHER

Guinea-Pigs	Previous Treatment	Re-injection (Intraperitoneal)	Reaction
1	11/2/14 0.1 c.c. horse serum 2 c.c. dog serum 11/10/14 1 c.c. horse serum (See Table 2, No. 1)	11/12/14 1 c.c. inactivated dog serum	Slight
2	11/2/14 0.1 c.c. horse serum 2 c.c. dog serum 11/2/14 1 c.c. horse serum (Table 2, No. 2)	11/21/14 1 c.c. inactivated dog serum	Very severe. Recovered
3	2/12/15 0.02 c.c. horse serum 2 c.c. human serum 3/1/15 1 c.c. horse serum (See Table 4, No. 4)	3/18/15 1 c.c. inactivated human serum	Death in 45 minutes
4	1/28/15 $\frac{1}{3}$ c.c. horse serum 2 c.c. dog serum 2/13/15 1 c.c. horse serum (See Table 5, No. 1)	2/16/15 1 c.c. inactivated dog serum	Slight
5	1/28/15 $\frac{1}{5}$ c.c. horse serum 2 c.c. of 50% egg-white solution 2/13/15 1 c.c. horse serum (See Table 5, No. 10)	2/29/15 1 c.c. of 50% egg-white solution	Very marked; prostration. Recovery
6	3/31/15 0.002 gm. egg albumin 2 c.c. dog serum	4/16/15 1 c.c. inactivated dog serum	Marked
7	3/31/15 0.0005 gm. egg albumin 2 c.c. dog serum	4/30/15 1 c.c. inactivated dog serum	Dead in 35 minutes

on the body cells of the guinea-pig so that they were unable to produce antibodies. To see whether toxicity was a factor, a non-toxic protein, egg-white, was used to replace the sera. Egg-white diluted one-half contains about the same amount of protein as dog serum. The experiment was conducted as follows:

On January 28, dilutions of horse serum were made so that 1 c.c. contained respectively 1/3 c.c., 1/6 c.c., 1/15 c.c., 1/75 c.c., 1/150 c.c., and 1/750 c.c. One cubic centimeter of each dilution was put into each of three test tubes so that there were three identical series of six test tubes each. To the tubes of one series were added 3 c.c. of fresh dog serum; to those of another, were added 3 c.c. of a 50 percent solution of whole egg-white. The mixtures were put into the incubator for two hours and into the ice-box for twelve hours. Three cubic centimeters from each tube were injected into guinea-pigs. The animals receiving 1/15 c.c. horse serum + 2 c.c. dog serum, and 1/750 c.c. horse serum + 2 c.c. dog serum, died during the night following the injection.

TABLE 7

THE INHIBITORY ACTION OF EGG-WHITE WHEN INJECTED PREVIOUSLY TO A SENSITIZING DOSE OF HORSE SERUM

Guinea-Pigs	Preliminary Treatment	Toxogenic Dose	Reaction
1	3/24/15 2 c.c. of a 5% solution egg albumin 3/25/15 2 c.c. of a 5% solution egg albumin 3/26/15 0.1 c.c. horse serum	2 c.c. horse serum (intraperitoneal) 4/13/15	Very slight
2	3/24/15 2 c.c. of a 5% solution egg albumin 3/25/15 2 c.c. of a 5% solution egg albumin 3/26/15 0.1 c.c. horse serum	2 c.c. horse serum (intraperitoneal) 4/13/15	Questionable
3	3/24/15 2 c.c. of a 5% solution egg albumin 3/25/15 2 c.c. of a 5% solution egg albumin 3/26/15 0.01 c.c. horse serum	2 c.c. horse serum (intraperitoneal) 4/13/15	None
4	3/24/15 2 c.c. of a 5% solution egg albumin 3/25/15 2 c.c. of a 5% solution egg albumin 3/25/15 0.001 c.c. horse serum	1 c.c. horse serum (intracardiac) 4/13/15	None
5	0.1 c.c. horse serum	2 c.c. horse serum (intraperitoneal) 4/13/15	Very severe
6	0.1 c.c. horse serum	2 c.c. horse serum (intraperitoneal) 4/13/15	Very severe
7	0.01 c.c. horse serum	2 c.c. horse serum (intraperitoneal) 4/13/15	Very severe
8	0.001 c.c. horse serum	1 c.c. horse serum (intracardiac) 4/13/15	Dead in 3 minutes

On February 13, all animals received intraperitoneally, horse serum diluted one-half. The results are seen in Table 5.

Egg-white, then, is just as efficient in inhibiting anaphylactic sensitization to horse serum as dog, cat, or human sera. Altho sensitization to horse serum is inhibited, there is sensitization to the inhibiting protein which is more marked after four weeks than after two weeks because of its large amount (see Table 6).

Egg-white will produce this inhibition, not only when mixed with the horse serum, but also when injected into the guinea-pigs before the horse serum is injected (described in Table 7).

In order to see whether there is any inhibition of the toxigenic action of an antigen by another protein, the following experiments were made:

On April 2, eight guinea-pigs received intraperitoneally 0.001 gm. Merck's egg albumin contained in 1 c.c. NaCl solution.

On April 15, the animals received intravenously varying amounts of egg albumin contained in 1 c.c. NaCl solution + 2 c.c. horse serum or 2 c.c. NaCl solution, with results as shown in Table 8.

TABLE 8
EFFECT OF HORSE SERUM ON THE TOXIGENIC ACTION OF EGG ALBUMIN

Guinea-Pigs	First Injection (Intraperitoneal)	Second Injection (Intravenous)	Reaction
1	0.001 gm. egg albumin	0.1 gm. egg albumin	Death immediately
2	0.001 gm. egg albumin	0.01 gm. egg albumin	Death in 5 minutes
3	0.001 gm. egg albumin	0.0001 gm. egg albumin	Death in 7.5 minutes
4	0.001 gm. egg albumin	0.0001 gm. egg albumin 2 c.c. horse serum	Death in 6.75 minutes
5	0.001 gm. egg albumin	0.0001 gm. egg albumin	Death in 7 minutes
6	0.001 gm. egg albumin	0.0001 gm. egg albumin 2 c.c. horse serum	Death in 5 minutes
7	0.001 gm. egg albumin	0.000001 gm. egg albumin	Marked. Recovery
8	0.001 gm. egg albumin	0.000001 gm. egg albumin 2 c.c. horse serum	Marked. Recovery

On April 19, eight guinea-pigs were given, intraperitoneally, 0.1 c.c. horse serum.

Later in April, the guinea-pigs were given varying amounts of horse serum + 2 c.c. of a 10 percent solution of Merck's egg albumin or 2 c.c. of NaCl solution mixed just before the injection. The results are seen in Table 9.

Recently Dr. H. J. Corper, in a personal communication, described experiments which were similar to those of Van Alstyne, made by

Dr. J. H. McClellan and himself in the physiological laboratories of the University of Illinois. Large quantities of egg-white were fed to dogs which were bled at regular intervals. The serum of these animals, even after the largest quantity of egg-white had been given, never sensitized guinea-pigs to egg-white. For this reason, the work was temporarily discontinued. At his request the next experiment was made.

TABLE 9
THE EFFECT OF EGG ALBUMIN ON THE TOXOGENIC ACTION OF HORSE SERUM

Guinea-Pigs	First Injection (Intraperitoneal)	Second Injection (Intravenous)	Reaction
1	1 c.c. horse serum	0.5 c.c. horse serum 2 c.c. NaCl	Dead in 10 minutes
2	1 c.c. horse serum	0.5 c.c. horse serum 0.2 gm. egg albumin	Dead in 8 minutes
3	1 c.c. horse serum	0.1 c.c. horse serum 2 c.c. NaCl	Dead in 35 minutes
4	1 c.c. horse serum	0.1 c.c. horse serum 0.2 gm. egg albumin	Dead in 43 minutes
5	1 c.c. horse serum	0.01 c.c. horse serum 2 c.c. NaCl	Very severe. Recovery
6	1 c.c. horse serum	0.01 c.c. horse serum 0.2 gm. egg albumin	Very severe. Recovery

On March 31, dilutions of egg albumin, crystallized by the Hopkins-Cole method, were made so that 1 c.c. contained respectively 0.005, 0.003, 0.001, and 0.0001 gm. of egg albumin. One cubic centimeter from each of these dilutions was mixed with 2 c.c. of fresh dog serum and another was mixed with 2 c.c. NaCl solution and injected into guinea-pigs intraperitoneally.

On April 16, each of the animals received 0.2 gm. egg albumin in 4 c.c. NaCl solution, intraperitoneally. The results are given in Table 10.

The results of this experiment are the most striking of all. All the control animals died, while those receiving dog serum with the sensitizing dose showed slight, if any, reaction.

In regard to another hypothesis that will be discussed farther on, an experiment was made to find the effect of egg-white on the passive sensitization of guinea-pigs. A rabbit was sensitized by repeated intraperitoneal injections of horse serum. At the end of two weeks, the rabbit was bled from the heart. Two series of guinea-pigs were each injected intraperitoneally with 2 c.c., 1 c.c. 0.3 c.c., and 0.1 c.c. of this serum. In one series, the serum was mixed with 2 c.c. of a 10 percent solution of Merck's egg albumin just before being injected

TABLE 10

THE INHIBITORY ACTION OF DOG SERUM ON ACTIVE SENSITIZATION WITH EGG ALBUMIN

Guinea-Pigs	First Injection	Reaction After Second Injection (Intraperitoneal) of 0.2 gm. Egg Albumin
1	0.005 gm. egg albumin 2 c.c. dog serum	Slight
2	0.003 gm. egg albumin 2 c.c. dog serum	Questionable
3	0.001 gm. egg albumin 2 c.c. dog serum	None
4	0.0001 gm. egg albumin 2 c.c. dog serum	None
5	0.005 gm. egg albumin 2 c.c. NaCl	Dead in 55 minutes
6	0.003 gm. egg albumin 2 c.c. NaCl	Dead in 28 minutes
7	0.001 gm. egg albumin 2 c.c. NaCl	Dead in 15 minutes
8	0.0001 gm. egg albumin 2 c.c. NaCl	Dead in 34 minutes

TABLE 11

THE EFFECT OF EGG PROTEIN ON PASSIVE SENSITIZATION

Guinea-Pigs	First Injection	Second Injection	Reaction
1	2 c.c. immune rabbit serum + 0.2 gm. egg albumin	0.2 c.c. horse serum (intravenous)	Very slight
2	2 c.c. immune rabbit serum	0.2 c.c. horse serum (intravenous)	Dead in 5 minutes
3	1 c.c. immune rabbit serum + 0.2 gm. egg albumin	0.2 c.c. horse serum (intravenous)	Slight
4	1 c.c. immune rabbit serum	0.2 c.c. horse serum (intravenous)	Very severe; died in 4 hours
5	0.3 c.c. immune rabbit serum + 0.2 gm. egg albumin	0.2 c.c. horse serum (intracardiac)	Slight
6	0.3 c.c. immune rabbit serum	0.2 c.c. horse serum (intracardiac)	Marked
7	0.1 c.c. immune rabbit serum + 2 gm. egg albumin	0.2 c.c. horse serum (intracardiac)	None
8	0.1 c.c. immune rabbit serum	0.2 c.c. horse serum (intracardiac)	Very slight

into the guinea-pigs. At the end of twenty-four hours each animal received 0.2 c.c. horse serum intravenously. As Table 11 shows, there was a marked inhibition of passive sensitization by means of the egg albumin. Also, as with active sensitization, passive sensitization was inhibited when the inhibiting protein was injected into the animal before the sensitizing protein (Table 12).

TABLE 12

THE INHIBITORY ACTION OF EGG ALBUMIN ON PASSIVE SENSITIZATION WHEN INJECTED BEFORE THE SENSITIZING SERUM

Guinea-Pigs	First Injection	Second Injection	Reaction After Third Injection (Intravenous), 24 Hours After the First, of 0.2 c.c. Horse Serum
1	0.2 gm. egg albumin	1 c.c. horse immune rabbit serum 24 hours after first injection	None
2	0.2 gm. egg albumin	1 c.c. horse immune rabbit serum 6 hours after first injection	Questionable
3	0.2 gm. egg albumin	1 c.c. horse immune rabbit serum 4 hours after first injection	None
4	0.2 gm. egg albumin	1 c.c. horse immune rabbit serum 2 hours after first injection	Very slight
5	0.2 gm. egg albumin 1 c.c. horse immune rabbit serum mixed together just before injection	0.2 c.c. horse serum (intravenous) 24 hours after first injection	None
6	1 c.c. horse immune rabbit serum + 2 c.c. NaCl solution	0.2 c.c. horse serum (intravenous) 24 hours after first injection	Very severe; prostrated. Recovery

The usual method of studying the absorption and persistence of antitoxin in the circulation is to inject a guinea-pig with a known amount of serum from the injected person or animal and then find how many M. L. D. of the toxin it can withstand. In order to see whether the action of antitoxin is inhibited by another protein as are anaphylactic antibodies, the next experiment was made. (I am much indebted to Dr. P. G. Heinemann for supplying me with standardized toxin and antitoxin and all the horse serum used in these experiments.)

Dilutions of a diphtheria toxin, the M. L. D. of which was 0.002 c.c. (for a guinea-pig weighing 250 gm.), were made so that each cubic centimeter contained 0.008 c.c. Of this dilution, 1.5 c.c. were measured accurately into each of twelve test glasses. Into four of these were measured 3 c.c. of fresh human

serum, heated to 56 C. for one-half hour, and into another four, were measured 3 c.c. of NaCl solution. The contents of a syringe containing approximately 2,000 units of diphtheria antitoxin were made into dilutions that contained in 1 c.c., 0.08 c.c., 0.04 c.c., 0.02 c.c., and 0.01 c.c. units of antitoxin. Of each of these four dilutions, 1.5 c.c. were measured into the test glasses containing human serum and toxin, or NaCl and toxin. To two other test glasses containing the same amount of toxin were added 1.5 c.c. NaCl solution and 3 c.c. human serum. To the two remaining test glasses with toxin, were added 4.5 c.c. NaCl. From each test glass 4 c.c. were measured into syringes, arranged so that they could be rinsed out, and the contents injected into the peritoneal cavity of guinea-pigs. The results are seen in Table 13.

TABLE 13
EFFECT OF HUMAN SERUM ON ANTITOXIC HORSE SERUM

Guinea-Pigs	Weight in Grams	Treatment	Results
1	361	0.01 unit antitoxin 2 c.c. NaCl solution 0.008 c.c. toxin	Dead in 1 day
2	374	0.01 unit antitoxin 2 c.c. horse serum 0.008 c.c. toxin	Dead in 4 days
3	391	0.02 unit antitoxin 2 c.c. NaCl solution 0.008 c.c. toxin	Dead in 2 days
4	390	0.02 unit antitoxin 2 c.c. horse serum 0.008 c.c. toxin	Dead in 6 days
5	390	0.04 unit antitoxin 2 c.c. NaCl solution 0.008 c.c. toxin	Dead in 7 days
6	402	0.04 unit antitoxin 2 c.c. horse serum 0.008 c.c. toxin	Dead in 10 days
7	418	0.08 unit antitoxin 2 c.c. NaCl solution 0.008 c.c. toxin	Lived
8	420	0.08 unit antitoxin 2 c.c. horse serum 0.008 c.c. toxin	Lived
9	436	2 c.c. horse serum 0.008 c.c. toxin	Dead in 4 days
10	440	2 c.c. horse serum 0.008 c.c. toxin	Dead in 4 days
11	382	0.008 c.c. toxin	Dead in 1 day
12	397	0.008 c.c. toxin	Dead in 1 day

Within the limits of accuracy of the method there was no perceptible effect of human serum on the antitoxic action of horse serum.

SUMMARY OF EXPERIMENTS

The blood or thoracic duct lymph of dogs injected with large amounts of horse serum subcutaneously, even under high pressure, will not sensitize guinea-pigs to horse serum.

Dog serum will inhibit almost completely the active sensitization of guinea-pigs by horse serum mixed with it before injection. Dog serum will also inhibit the active sensitization of guinea-pigs by egg protein mixed with it before injection.

Cat and human sera, and egg protein will inhibit active sensitization by horse serum mixed with them, in no way differently from dog serum.

Horse serum also inhibits active sensitization by egg protein.

Egg protein inhibits active sensitization to horse serum if injected before the sensitizing dose of horse serum.

Horse serum has no effect on the toxigenic action of egg protein in animals sensitized with egg protein, and egg protein has no effect on the toxigenic action of horse serum in animals sensitized with horse serum.

Egg protein inhibits the passive sensitization of guinea-pigs with horse-immune rabbit serum when injected either with or before the sensitizing serum.

Human serum has no effect on the protective action of antitoxic horse serum.

DISCUSSION

In pursuit of an explanation of the phenomena thus described, our experiments have assumed a similarity to those performed by Weil,⁴ and ideas expressed by him have been helpful in explaining our results. He has shown that if a guinea-pig is given several injections of comparatively large amounts of normal rabbit or sheep serum, it cannot be passively sensitized to horse serum with horse-immune rabbit serum one to fourteen days after the preliminary treatment. Moreover, guinea-pigs given a sensitizing dose of horse-immune rabbit serum will not be sensitive to horse serum six to nine days later if the sensitizing dose is followed by several injections of normal rabbit or sheep serum. Weil suggests two possible explanations for these results. In the first place, the normal sera, when given before the sensitizing serum, may "saturate" the cell receptors of the body, leaving none available to anchor the immune rabbit serum; and when given

4. Jour. Med. Research, 1913, 28, p. 243.

after the injection of immune serum, they may "displace" the immune bodies. Or, for another possible explanation, the normal sera may give rise to antibodies which prevent the union of antigen and antibody. He did not determine the effect of these proceedings on active sensitization.

Altho Weil is more inclined to accept the latter view as correct, the first one, i. e., "saturation" or "displacement," is more in harmony with our work. The number of cell receptors in the body must be limited and therefore the ability of the cells to take up foreign proteins is limited. Likewise, the capacity of each cell to produce immune bodies or sessile receptors is limited. If every antibody-producing cell in the body were stimulated by an optimal amount of foreign protein, the number of antibodies formed would be the product of the number of antibody-forming cells multiplied by the working capacity of each cell. If the antigen was two chemically distinct proteins in equal amounts, the total number of antibodies formed would be theoretically the same, but the number for each protein would be just one-half because the number of active cells stimulated by each protein is one-half the total number, or, if one cell, having a limited working capacity, can fix more than one protein, the output of each one is reduced one-half.

In our experiments, according to this idea, then, there is inhibition of anaphylaxis for the smaller dose of protein because it was prevented by the protein in excess from uniting in sufficient amount, or was prevented from uniting with a sufficient number of cell receptors, to produce maximal sensitization.

If this is true, the amount of inhibition should depend on the ratio of the amounts of the two proteins injected. All ranges of inhibition should be obtained by varying their proportion. This was put to test experimentally.

Ten guinea-pigs received 0.1 c.c. horse serum intraperitoneally. Together with the horse serum, eight of them received respectively 2 c.c., 1.5 c.c., 1 c.c., 0.5 c.c., 0.25 c.c., 0.1 c.c., 0.01 c.c., and 0.001 c.c. of dog serum. Fourteen days later all received 2 c.c. normal horse serum intraperitoneally. The results are seen in Table 14.

There has been nearly absolute inhibition in the guinea-pigs receiving 2 c.c., 1.5 c.c. and 1 c.c. dog serum. There was some noticeable difference between the one that received only 0.001 c.c. dog serum and the controls.

TABLE 14

THE INHIBITORY EFFECT OF VARYING AMOUNTS OF DOG SERUM ON ACTIVE SENSITIZATION TO HORSE SERUM

Guinea-Pigs	First Injection	Reaction After Second Injection of 2 c.c. Horse Serum Diluted One Half
1	0.1 c.c. horse serum 2 c.c. dog serum	Scratched nose occasionally. Doubtful
2	0.1 c.c. horse serum 1.5 c.c. dog serum	None
3	0.1 c.c. horse serum 1 c.c. dog serum	None
4	0.1 c.c. horse serum .5 c.c. dog serum	Marked; died after 50 hours
5	0.1 c.c. horse serum .25 c.c. dog serum	Severe. Recovery
6	0.1 c.c. horse serum .1 c.c. dog serum	Very severe. Recovery
7	0.1 c.c. horse serum .01 c.c. dog serum	Severe. Recovery
8	0.1 c.c. horse serum .001 c.c. dog serum	Severe; died after about 10 hours
9	0.1 c.c. horse serum	Died in 1 hour
10	0.1 c.c. horse serum	Died in 50 minutes

In the passive anaphylaxis experiments, the immune bodies are "shunted" off from the cells that fix them by the large amount of egg albumin. The two proteins, immune rabbit serum and egg albumin, having an equal chance at the cell receptors, are anchored in proportion to their quantities. The "shunting off," as would be expected, occurs if the egg albumin is injected before, or at the same time as, the injection of the immune rabbit serum. On the other hand, anti-toxin does not require binding to the body cells to afford protection. Probably only the antitoxin free in the circulation can be efficient. Hence, from our thesis, it would be expected, as was found, that other heterologous proteins, in whatever amounts, would not inhibit the combining of the toxin with its specific antitoxin.

The fact that the inhibition of passive anaphylaxis occurs if the egg albumin is injected with the immune serum is serious evidence against the idea that antibodies are formed which prevent anaphylactic intoxication, because it would be necessary that these antibodies be formed in twenty-four hours, and it cannot be due to "displacement" because the immune bodies are presumably not yet "placed."

It is admitted that a serious objection to the acceptance of this explanation is that it is against our ideas of specificity. It suggests that two proteins may be anchored by the same receptors. This objection may not be serious. If an antigen can combine only with a specific receptor, it means that the body cells must contain the unbelievable number of receptors that would be required to bind every protein that occurs in nature, and in addition, a large number of modifications of these proteins by various changes produced in the "constitutive Gruppierung." Non-specific antibodies are known to occur normally in the body. Normal human serum not only inhibits proteolysis by homologous enzymes, but also, and even more so, by enzymes of the pig and perhaps other animals.⁵

In these experiments may lie the explanation of the observation made by Wells² that sensitization could not be obtained with as small an amount of whole egg-white as of pure, crystallized egg albumin. With whole egg-white the antibody formation is divided between four chemically and biologically distinguishable antigens.

A number of investigators have shown that antigen rests exist in the circulation of immune animals, by showing that the serum of these animals will actively sensitize another one to the antigen. Gay and Southard⁶ showed that the serum of a guinea-pig immune to horse serum will sensitize another guinea-pig within fifteen days to horse serum. Hintze⁷ gave rabbits a single relatively large dose of horse serum or egg-white, bled them at regular intervals, and injected the serum into guinea-pigs. The guinea-pigs were sensitized to horse serum but not markedly so. The same experiment was made by Jonesco-Mihaiesti,⁸ except that still larger doses of antigen were given the rabbits in repeated injections. The same positive results were obtained. The method of Gay and Southard determined more accurately the true amount of antigen rest, because the immune serum was injected into a homologous animal and the inhibitory action of the heterologous serum was not obtained as in the experiments of Hintze and Jonesco-Mihaiesti. That the latter obtained active sensitization at all with the immune serum is probably due to the fact that the rabbits were immunized with relatively large doses of antigen, and enough was retained in the circulation to overcome the inhibition of the rabbit serum.

5. Von Eisler, *Ber. d. Wien. Akad.*, 1905, 104, p. 119.

6. *Jour. Med. Research*, 1907, 16, p. 143.

7. *Ztschr. f. Immunitätsforsch.*, 1910, 6, p. 113.

8. *Compt. rend. Soc., de biol.*, 1911, 70, p. 104.

A number of investigators have sensitized animals with two or more proteins in the study of the specificity of antianaphylaxis. As controls were not made in these experiments by sensitizing animals with the same amount of one protein alone, the mutual inhibition of anaphylactic sensitization to the several proteins was apparently not observed. But our attention has been called to the work of Benjamin and Witzinger⁹ who have made experiments exactly similar to some of ours in active sensitization. They found that a large dose of horse serum would inhibit the sensitization of a guinea-pig to beef serum given at the same time or twenty-four hours later. They also found that a preliminary injection of horse serum would inhibit the formation of hemolysins by rabbits for sheep corpuscles and that a large dose of beef serum would inhibit the formation by rabbits of precipitins for horse serum, thus giving further support to the idea of saturation of non-specific receptors as the cause of inhibition.

GENERAL SUMMARY

An amount of protein that will produce a marked anaphylactic sensitization when injected alone into a guinea-pig, will fail to do so if injected together with, or twenty-four hours after, a much larger amount of another protein.

The serum from a rabbit immune to horse serum, which will markedly sensitize a guinea-pig to horse serum, will fail to do so if injected with, or twenty-four hours after, a large dose of another protein.

These results may be explained by the conception that the number of receptors in the body that can unite with a foreign protein is limited. The inhibiting protein, if present in large amount, combines with all, or almost all, of these receptors. Hence, another protein injected with it, or after it, is prevented from being combined in sufficient amount to stimulate the active production of antibodies. And when a large amount of protein is injected with or after a sensitizing dose of immune serum, the combination of the latter with the cell receptors, which is necessary for passive sensitization, is prevented in the same way.

9. *Ztschr. f. Kinderheilk.*, 1911, 3, p. 73.

THE ANAPHYLACTIC REACTION WITH SO-CALLED PROTEOSES OF VARIOUS SEEDS

THE BIOLOGIC REACTIONS OF THE VEGETABLE PROTEINS, VI *

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Altho the toxicity of proteoses and peptones derived from animal tissues has been extensively investigated,¹ their antigenic capacity has received relatively little attention. Few useful conclusions can be drawn from most of this work since it has been done chiefly with Witte's "peptone." As the composition of this commercial product is uncertain, the results which have been reported are of doubtful value. (Zunz states that different samples of Witte's "peptone" may vary in the proportion of proteose nitrogen from 47.52 to 83.21 percent.) Since the literature of this subject has been thoroughly reviewed by Zunz,² the results recorded may here be summarized briefly by stating that (1) as far as the precipitin reaction is concerned, negative results have been obtained with peptone and proteose preparations; (2) we can find no statements concerning complement-fixation antibodies for substances of this class; (3) the attempted demonstration of anaphylactogenic properties in proteoses derived from animal proteins has usually given negative results. One of us has studied all the different fractions obtainable from tryptic and peptic digestion of egg-white, both coagulated and raw, and has been unable to find any evidence whatever that its proteoses, peptones, or any of the lower products of hydrolysis, can sensitize guinea-pigs either to egg-white or to the digestion products themselves.³ Such fragments do not cause intoxication of animals sensitized with egg-white, nor do they prevent reactions in such animals when egg-white is subsequently injected. The supposedly protec-

* Received for publication May 28, 1915.

The expenses of this investigation were shared by the Carnegie Institution of Washington, D. C.

The previous papers of this series were published in *The Journal of Infectious Diseases*, as follows: I, 1911, 8, p. 66; II, 1913, 12, p. 341; III, 1913, 13, p. 103; IV, 1914, 14, p. 364; V, 1914, 14, p. 377.

1. See review by Gibson, *Philippine Jour. Sc.*, 1913, 8, p. 475.

2. *Ztschr. f. Immunitätsforsch.*, 1913, 16, p. 580.

3. Wells, *Jour. Infect. Dis.*, 1909, 6, p. 506.

tive action, described by Biedl and Kraus,⁴ who used Witte's peptone in dogs, was therefore not exhibited. From these observations, and others in which various steps in the enzymatic disintegration of proteins had been studied by the reaction of anaphylaxis, the conclusion was drawn that, as far as anaphylaxis is concerned, nothing less than entire protein molecules can act as antigens.

The only observations to the contrary are those of Zunz and of Abderhalden. Zunz⁵ states that he produced both active and passive anaphylaxis in guinea-pigs and rabbits with hetero-albumose, proto-albumose, and synalbumose, but that synalbumose is only able to sensitize. Negative results were obtained with all the other products of proteolysis, including thio-albumose, "secondary proteoses," Siegfried's pepsin-fibrin-peptone β , as well as the lower abiuret products. The hetero-albumose was generally more effective than the proto-albumose. This work has been in part repeated by Friedberger and Joachimoglu,⁶ who, using the same preparations of proto-albumose and hetero-albumose that Zunz had employed, found no anaphylactogenic properties in these proteoses: they neither sensitized to serum, nor caused reactions in animals when used for both the intoxicating and sensitizing doses. These authors point out errors in Zunz's work which they believe account for his supposedly positive results.

Abderhalden reports that he has obtained typical anaphylaxis reactions with the fraction of "silk peptone" precipitated with ammonium sulphate, but not with the fractions which this salt failed to precipitate. With a polypeptid containing fourteen radicals of amino-acids (including only glycin and leucin), he observed a fall of temperature, and, in another experiment, a more severe reaction, with convulsions and death (after six hours!). Since the details of this experiment are omitted, a critical consideration is impossible.

In many grains and seeds are found protein substances which, on account of their solubilities, have been designated as "proteoses." As to the immunologic relations of these naturally occurring vegetable proteoses, we have found only the following:

Schloss⁸ studied a boy suffering from an urticarial eruption whenever he ate eggs, almonds, or oatmeal. Experiments showed that of the various preparations from almonds and oatmeal, the most active in producing local skin reactions in this subject were the "proteoses" of these seeds, possibly because of their greater solubility.

Schneider⁹ found the strong hemagglutinative principle of *Phaseolus* to be a proteose which disappears during germination.

DeWaele¹⁰ ascribes toxic properties, similar to those of albumoses in general, to a product obtained by heating wheat-gluten with dilute hydrochloric acid.

4. Wien. klin. Wchnschr., 1909, 22, p. 363.

5. Ztschr. f. Immunitätsforsch., 1913, 16, p. 580.

6. Ibid., 1914, 22, p. 522.

7. Ztschr. f. physiol. Chem., 1912, 81, p. 315.

8. Amer. Jour. Dis. Child., 1912, 3, p. 341.

9. Jour. Biol. Chem., 1912, 11, p. 47.

10. Ztschr. f. Immunitätsforsch., 1912, 13, p. 644.

These few observations on the antigenic power of animal and vegetable proteoses have led us to study the immunologic relations of the so-called proteoses obtained from seeds, and to compare them with proteoses made by artificial methods from the more abundant proteins of some of the same seeds.

Extracts of practically all seeds thus far examined contain a small amount of protein which cannot be separated by prolonged dialysis in water, nor by heating for a long time at 100 C. Since the solubility of this protein corresponds to that of the proteoses produced by the action of proteolytic enzymes on native proteins, they have been called vegetable "proteoses." It is not known that any chemical relation exists between these vegetable "proteoses" and those produced by enzymes, for none of them has ever been obtained in sufficient quantity to make a satisfactory comparison possible. We do not know therefore whether these vegetable "proteoses" have as complex a structure as the native proteins, or a simpler one, corresponding to the proteoses formed from native proteins by hydrolysis. Furthermore, we do not know whether these "proteoses" are original constituents of the seed, or are formed during the processes incident to their separation from the extract. It is probable that enzymatic decomposition of the reserve protein of many seeds takes place during extraction, notably in the case of the flax-seed¹¹ and lupine-seed,¹² and in such cases preparations of "protease" from these seeds doubtless consist largely of hydrolytic decomposition products of the reserve protein.

Some seeds contain protein which can be separated by dialysis from the extract only when this is slightly acid, i. e., the greater part of their reserve protein behaves like a globulin when combined with a small proportion of acid, and much like a proteose when free from combined acid, for in this condition it is very imperfectly coagulated by heat. In such cases it is difficult, if not impossible, to separate the reserve protein from the proteose. Examples of such seeds are the almond, hazel-nut, and walnut (*Juglans*).

From these statements it is evident that preparations of the natural vegetable "proteoses" are of uncertain chemical nature, and that they require much more careful study than they have as yet received, before any definite characteristics can be assigned to them.

In order to determine the anaphylactogenic activity of these "proteoses," and their immunologic relations to the reserve proteins of the

11. Osborne, Am. Chem. Jour., 1892, 14, p. 629.

12. Osborne and Harris, Am. Jour. Physiol., 1905, 13, p. 436.

seeds from which they were obtained, we have made the experiments reported in the following pages. The results described in this paper must be regarded as preliminary to a more precise study, because most of the preparations used were by-products obtained in previous investigations with entirely different objects, and were not sufficiently purified to make them suitable for final experiments of the kind here reported. The results of this preliminary survey furnish a basis for further study, and it is our intention, in the near future, to repeat the experiments here described, using more carefully purified preparations made especially for the work.

In all cases the injections were made intraperitoneally, and the terms indicating the severity of the reactions have the same significance as in previous articles.¹³ The nature and results of these experiments are summarized in the following tables:

TABLE 1
"PROTEOSE," CASTOR-BEAN, RICINUS ZANZIBARENSIS

Experiment	Sensitizing Dose	Second Dose	Result	Protection Test*
1	0.01 gm. proteose, castor bean	0.05 gm. proteose, castor bean	Died, 60 min.	
2	0.01 gm. proteose, castor bean	0.03 gm. proteose, castor bean	Died, 6 min.	
3	0.01 gm. proteose, castor bean	0.05 gm. globulin, castor bean	Doubtful....	None; died in 1 hour
4	0.01 gm. proteose, castor bean	0.05 gm. globulin, castor bean	Doubtful....	None; died in 20 minutes
5	0.01 gm. globulin, castor bean	0.05 gm. proteose, castor bean	Slight.....	None; severe reaction
6	0.01 gm. globulin, castor bean	0.05 gm. proteose, castor bean	Slight.....	None; severe reaction
7	0.05 gm. proteose, castor bean	0.05 gm. globulin, castor bean	0	None; died in 1 hour

* The term "Protection Test" is applied to the reaction obtained after saturating the guinea-pig with the heterologous protein, and then injecting with the homologous protein. "None" means no protection, the animal still being strongly sensitive to the protein used for sensitizing.

The preparation of "proteose" used in these experiments was obtained in an extensive fractionation of the water-soluble proteins of the castor-bean, the purpose of which was to separate ricin in as pure a condition as possible. No evidence was obtained indicating enzymatic decomposition of the reserve protein in the extracts of this seed, and it is probable that the "proteose" was preformed in this seed. This preparation, which represents only a fraction of the total "proteose," was separated from the other proteins by dialyzing the sodium chlorid extract of the seed until all of the globulin had separated, and

then subjecting the solution to fractional precipitation with alcohol. The extract was not heated during the process of isolation.

These results are of interest as showing (a) the high anaphylactogenic power of the "protease"; (b) the practically complete separation from ricin, for 50 milligrams, used for the sensitizing dose (Exper. 7), caused no symptoms of ricin poisoning, and 100 milligrams killed a guinea-pig weighing 400 grams only after ten days, whereas a preparation consisting chiefly of the albumin from the same extract which yielded this "protease," killed rabbits in doses of 0.002 milligram per kilogram within three and one-half days; (c) the complete separation of globulin from the "protease," for animals sensitized with even 50 milligrams gave no reaction when subsequently injected with the

TABLE 2
"PROTEOSE," FLAX-SEED, LINUM USITATISSIMUM

Experiment	Sensitizing Dose	Second Dose	Result	Protection Test
1	0.01 gm. proteose, flax-seed	0.10 gm. proteose, flax-seed	Died, 60 min.	
2	0.005 gm. proteose, flax-seed	0.10 gm. proteose, flax-seed	Severe, died during night	
3	0.001 gm. proteose, flax-seed	0.10 gm. proteose, brazil-nut	0	None; severe reaction
4	0.001 gm. proteose, flax-seed	0.05 gm. globulin, flax-seed	Slight.....	Partial; slight reaction
5	0.005 gm. proteose, flax-seed	0.05 gm. globulin, flax-seed	Moderate....	Partial; slight reaction
6	0.010 gm. globulin, flax-seed	0.05 gm. proteose, flax-seed	Moderate....	None; severe reaction
7	0.010 gm. globulin, flax-seed	0.05 gm. proteose, flax-seed	Slight, died during night	
8	0.001 gm. globulin, flax-seed	0.05 gm. proteose, flax-seed	Died, 1 hr. 30 min.	
9	0.010 gm. proteose, flax-seed	0.10 gm. proteose, hemp-seed	0	

globulin; (d) evidence of a trace of "protease" in the preparation of the globulin, for animals sensitized with 0.01 gm. of the globulin gave a slight reaction after a second dose of 0.05 gm. of "protease." This trace of "protease" was probably adsorbed during precipitation of the globulin, and doubtless could have been removed if the globulin had been reprecipitated several times.

Whether this "protease" is an original constituent of the seed, or a product of hydrolysis of the globulin is not known, but its anaphylactogenic activity indicates that it is not a hydrolytic product. That in this preparation the separation from the globulin was not complete is indicated by the slight to moderate reaction obtained when the globulin is administered to guinea-pigs sensitized with the "protease." The presence of some "protease" in the preparation of globulin is shown

by the experiments made in the reverse order. Since the globulin was precipitated by dialysis from the extract containing the "proteose," and as this preparation had not been especially purified by repeated precipitations, it is probable that a little "proteose" was adsorbed by the globulin during precipitation.

Unpublished experiments by one of us (O.) have made it very probable that a part, at least, of the "proteose" obtained from extracts of the hemp-seed is derived from edestin through changes that occur during the process of isolation. Under such circumstances edestin preparations might adsorb some of the proteose-like products which

TABLE 3
"PROTEOSE," HEMP-SEED, CANNABIS SATIVA

Experi- ment	Sensitizing Dose	Second Dose	Result	Protection Test
1	0.01 gm. proteose, hemp-seed	0.05 gm. proteose, hemp-seed	Died, 30 min.	
2	0.01 gm. proteose, hemp-seed	0.10 gm. edestin, hemp-seed	Slight.....	None; severe reaction
3	0.01 gm. proteose, hemp-seed	0.10 gm. edestin, hemp-seed	Severe.....	Partial; moderate reaction
4	0.01 gm. proteose, hemp-seed	0.10 gm. edestin, hemp-seed	Slight.....	None; severe reaction
5	0.01 gm. proteose, hemp-seed	0.10 gm. edestin, hemp-seed	Slight.....	Partial; moderate reaction
6	0.005 gm. proteose, hemp-seed	0.10 gm. edestin, hemp-seed	0	Partial; slight reaction
7	0.001 gm. proteose, hemp-seed	0.10 gm. edestin, hemp-seed	0	Partial; slight reaction
8	0.010 gm. edestin, hemp-seed	0.05 gm. proteose, hemp-seed	Slight.....	None; moderate reaction
9	0.010 gm. edestin, hemp-seed	0.05 gm. proteose, hemp-seed	Slight.....	
10	0.010 gm. proteose, hemp-seed	0.05 gm. globulin, squash-seed	0	

are formed. The slight reactions obtained between edestin and hemp-seed "proteose" may be thus explained. The much stronger reactions produced by sensitizing and intoxicating with the so-called proteose are doubtless caused by a protein distinctly different from edestin in chemical constitution rather than by the greater solubility of the "proteose" preparations in water.

These experiments show that this preparation of proteose has been so completely separated from the globulin that its presence cannot be detected by this very delicate reaction, and that the "proteose" of the squash-seed is a distinctly different protein from the globulin. The moderate to doubtful reactions shown by guinea-pigs sensitized with the globulin and intoxicated with the "proteose" is to be ascribed to a natural toxicity of the "proteose" itself, demonstrated by control

experiments, rather than to "protease" adsorbed by the globulin during precipitation, for if the latter were the case some protection should have been afforded.

Excelsin separates from slightly acid solutions in beautiful hexagonal crystals, which are practically insoluble in pure water, but soluble in saline solutions. The crystals are compounds of excelsin with acid, i. e., a protein salt. If the acid is removed by being neutralized with alkali, excelsin is then soluble in pure water. Unless sufficient acid is present to convert all of the excelsin into the compound insoluble in water, more or less remains in solution when the

TABLE 4
"PROTEOSE," SQUASH-SEED, CUCURBITA MAXIMA

Experiment	Sensitizing Dose	Second Dose	Result	Protection Test
1	0.01 gm. proteose, squash-seed	0.05 gm. proteose, squash-seed	Died, 8 min.	
2	0.01 gm. proteose, squash-seed	0.05 gm. proteose, castor bean	0	
3	0.01 gm. proteose, squash-seed	0.05 gm. globulin, squash-seed	0	None; severe reaction
4	0.01 gm. proteose, squash-seed	0.05 gm. globulin, squash-seed	0	None; died
5	0.001 gm. proteose, squash-seed	0.05 gm. globulin, squash-seed	0	
6	0.001 gm. proteose, squash-seed	0.05 gm. globulin, squash-seed	0	None; severe reaction
7	0.010 gm. globulin, squash-seed	0.05 gm. proteose, squash-seed	Moderate....	None; severe reaction
8	0.010 gm. globulin, squash-seed	0.05 gm. proteose, squash-seed	Moderate....	None; severe reaction
9	0.001 gm. globulin, squash-seed	0.05 gm. proteose, squash-seed	Doubtful....	None; died
10	0.001 gm. globulin, squash-seed	0.05 gm. proteose, squash-seed	Doubtful....	None; died

salts are removed by dialysis. For this reason it is difficult to separate all of the excelsin from the "protease." The results of these experiments indicate that this preparation of "protease" contained some excelsin. The very great anaphylactic intoxicating power of the "protease," shown by these experiments, demands a very complete separation of the "protease" from the excelsin if no reaction is to be given by animals sensitized with excelsin and intoxicated by the "protease." That this separation was not quite complete is shown by Experiments 11-19. Investigations of the proteins of the Brazil-nut indicate that this seed contains a relatively considerable quantity of preformed "protease," and the results of the experiments here given show that this is biologically, and therefore chemically, distinct from excelsin.

On account of the evident admixture of excelsin with our preparation of "Brazil-nut protease," an attempt was made to purify it further.

Six grams were shaken thoroughly with distilled water and filtered after standing twelve hours on ice. A perfectly clear filtrate was obtained, and about 2 gm. of insoluble residue were left undissolved. The protein in the filtrate was precipitated with alcohol, and dried with alcohol and ether, about 4 gm. of material being obtained. Two guinea-pigs sensitized with excelsin were given each 0.05 gm. of this preparation; one died in twenty-eight minutes, the other nearly died. Two guinea-pigs sensitized with 0.001 gm. of this "protease" reacted severely to 0.05 gm. excelsin, one dying in the night. The minimal

TABLE 5
"PROTEOSE," BRAZIL-NUT, BERTHOLLETIA EXCELSA

Experiment	Sensitizing Dose	Second Dose	Result	Protection Test
1	0.01 gm. proteose, brazil-nut	0.10 gm. proteose, brazil-nut	Died, 15 min.	
2	0.0005 gm. proteose, brazil-nut	0.10 gm. proteose, brazil-nut	Died, 20 min.	
3	0.002 gm. proteose, brazil-nut	0.10 gm. proteose, flax-seed	0	None; died, 8 minutes
4	0.001 gm. proteose, brazil-nut	0.10 gm. proteose, flax-seed	0	None; died, 8 minutes
5	0.001 gm. proteose, brazil-nut	0.001 gm. proteose, brazil-nut	Died, 12 min.	
6	0.001 gm. proteose, brazil-nut	0.002 gm. proteose, brazil-nut	Died, 12 min.	
7	0.001 gm. proteose, brazil-nut	0.0005 gm. proteose, brazil-nut	Died, 80 min.	
8	0.001 gm. proteose, brazil-nut	0.0005 gm. proteose, brazil-nut	Died, 70 min.	
9	0.001 gm. proteose, brazil-nut	0.0001 gm. proteose, brazil-nut	Severe	
10	0.001 gm. proteose, brazil-nut	0.00005 gm. proteose, brazil-nut	Slight.....	Killed by 0.05 gm. excelsin
11	0.005 gm. proteose, brazil-nut	0.05 gm. excelsin, brazil-nut	Severe.....	Nearly complete; slight reaction
12	0.001 gm. proteose, brazil-nut	0.05 gm. excelsin, brazil-nut	Severe.....	Nearly complete; slight reaction
13	0.0005 gm. proteose, brazil-nut	0.05 gm. excelsin, brazil-nut	Severe, died in night	
14	0.05 gm. proteose, brazil-nut	0.10 gm. excelsin, brazil-nut	Died, 1 hr.	
15	0.001 gm. proteose, brazil-nut	0.10 gm. excelsin, brazil-nut	Died, 45 min.	
16	0.01 gm. excelsin, brazil-nut	0.05 gm. proteose, brazil-nut	Slight.....	Partial; moderate reaction
17	0.01 gm. excelsin, brazil-nut	0.05 gm. proteose, brazil-nut	Slight.....	Partial; moderate reaction
18	0.001 gm. excelsin, brazil-nut	0.05 gm. proteose, brazil-nut	Died, 6 min.	
19	0.001 gm. excelsin, brazil-nut	0.05 gm. proteose, brazil-nut	Severe	

lethal dose of this preparation for 300 gm. guinea-pigs sensitized with it was between 0.001 gm. and 0.002 gm. Since this preparation evidently still contained excelsin, the process was repeated four times more, using only about 50 to 100 c.c. water for each re-solution. Each time there was some material that dissolved only slowly in water, and this was discarded as far as possible. Finally about 0.5 gm. of material was left which was readily soluble in 50 c.c. of water, but even this preparation sensitized fatally to excelsin, and caused severe reactions in guinea-pigs sensitized with excelsin. These experiments show that it is very difficult, if not impossible, to separate excelsin and "protease" completely.

A series of experiments was also performed in which the effect of heating the unpurified Brazil-nut "protease" was examined. The "protease" was sus-

pended in water and heated one-half hour at 100 C.; after the suspension had cooled, sodium hydrate was added to make 0.1 percent, but the "protease" did not completely dissolve. It was found that with this heated "protease" fatal results were produced in sensitized guinea-pigs with doses of 0.002 to 0.005 gm., and, of two guinea-pigs given 0.001 gm., one died after several hours, while the other was only moderately intoxicated; 0.0005 gm. doses caused but slight

TABLE 6
"PROTEOSE," BLACK-WALNUT, *JUGLANS NIGRA*

Experiment	Sensitizing Dose	Second Dose	Result
1	0.01 gm. proteose, walnut.....	0.05 gm. proteose, walnut.....	Died, 45 minutes
2	0.01 gm. proteose, walnut.....	0.05 gm. proteose, walnut.....	Severe reaction
3	0.01 gm. proteose, walnut.....	0.05 gm. globulin, walnut.....	Severe reaction
4	0.01 gm. proteose, walnut.....	0.05 gm. globulin, walnut.....	Died, 90 minutes
5	0.01 gm. globulin, walnut.....	0.05 gm. proteose, walnut.....	Severe reaction
6	0.01 gm. globulin, walnut.....	0.05 gm. proteose, walnut.....	Severe reaction

to moderate symptoms. As 0.0005 gm. doses of the unheated "protease" were found regularly fatal, it is evident that heating does somewhat impair the anaphylactic toxicity of this "protease," altho the effect of heat is relatively slight, as compared with the effect of heat on coagulable proteins, and may well be ascribed to the coagulation of the excelsin present in this preparation.

TABLE 7
"PROTEOSE," HAZEL-NUT, *CORYLUS AVELLENA*

Experiment	Sensitizing Dose	Second Dose	Result	Protection Test
1	0.005 gm. proteose, hazel-nut	0.05 gm. proteose, hazel-nut	Died, 15 min.	
2	0.005 gm. proteose, hazel-nut	0.05 gm. proteose, lentil	0	None; severe reaction
3	0.005 gm. proteose, hazel-nut	0.05 gm. proteose, lentil	0	Partial; moderate reaction
4	0.010 gm. proteose, hazel-nut	0.05 gm. corylin, hazel-nut	Severe.....	None; severe reaction
5	0.010 gm. proteose, hazel-nut	0.05 gm. corylin, hazel-nut	Died, 30 min.	
6	0.010 gm. corylin, hazel-nut	0.05 gm. proteose, hazel-nut	Doubtful....	Complete; no reaction
7	0.010 gm. corylin, hazel-nut	0.05 gm. proteose, hazel-nut	Slight.....	Complete; slight reaction
8	0.001 gm. proteose, hazel-nut	0.001 gm. proteose, hazel-nut	Slight	
9	0.001 gm. proteose, hazel-nut	0.002 gm. proteose, hazel-nut	Slight	
10	0.001 gm. proteose, hazel-nut	0.003 gm. proteose, hazel-nut	Slight	
11	0.001 gm. proteose, hazel-nut	0.004 gm. proteose, hazel-nut	Severe, died in night	

As previously mentioned, juglansin, the globulin found in different species of walnuts, separates on dialyzing the sodium chlorid extract of the seed only when this contains a certain small proportion of acid. For this reason it is very difficult to obtain from this seed preparations of "protease" which are free from globulin. The preparation used for

these experiments evidently contained a relatively large amount of the globulin.

This preparation of "protease" from the hazel-nut, which apparently has less anaphylactogenic power than the natural "protease" of the Brazil-nut, evidently contained enough of the globulin, corylin, to sensitize the animals to corylin, but not enough to intoxicate animals sensitized with corylin. The preparation of corylin appears, therefore, to be nearly free from "protease," but the "protease" is not free from corylin. The imperfect separation of corylin from the "protease" can

TABLE 8
"PROTEOSE" AND "PROTOPROTEOSE," PEA, *PISUM SATIVUM*

Experiment	Sensitizing Dose	Second Dose	Result	Protection Test
1	0.01 gm. proteose, pea	0.05 gm. proteose, pea	Severe.....	Nearly complete
2	0.01 gm. proteose, pea	0.04 gm. protoproteose, pea	Severe.....	Nearly complete
3	0.01 gm. proteose, pea	0.10 gm. legumin, pea	Slight.....	Slight or none
4	0.01 gm. proteose, pea	0.10 gm. legumin, pea	Slight.....	Slight or none
5	0.01 gm. protoproteose, pea	0.05 gm. proteose, pea	Severe.....	Partial
6	0.01 gm. protoproteose, pea	0.05 gm. protoproteose, pea	Died, 3 min.	
7	0.01 gm. protoproteose, pea	0.05 gm. proteose, pea	Severe.....	Partial
8	0.01 gm. protoproteose, pea	0.10 gm. legumin, pea	Doubtful...	Partial
9	0.01 gm. vicillin, pea	0.05 gm. proteose, pea	Doubtful...	Partial
10	0.01 gm. vicillin, pea	0.05 gm. proteose, pea	0	Partial
11	0.01 gm. vicillin, pea	0.05 gm. protoproteose, pea	Moderate...	Partial
12	0.01 gm. legumin, pea	0.05 gm. proteose, pea	0	Partial
13	0.01 gm. legumin, pea	0.05 gm. proteose, pea	Doubtful...	Partial
14	0.01 gm. legumin, pea	0.05 gm. protoproteose, pea	Slight.....	Partial
15	0.01 gm. legumin, pea	0.05 gm. protoproteose, pea	Moderate...	Partial

be attributed to the same cause as that assigned to the incomplete separation of excelsin or juglansin from the "proteoses" of the Brazil-nut and walnut, respectively. These experiments show that corylin is chemically distinct from the "protease."

The preparation of "protoproteose" was made by the conventional methods formerly used in separating mixtures of proteoses into fractions, to which distinguishing names have been assigned, e. g., proto-, deuter-, etc., proteose. Thus, the total protein extracted from the seed was precipitated by saturating with ammonium sulphate, the solution of the precipitate freed from globulin by long dialysis, the albumins separated from the filtrate by heating to 95 C., and the total "protease" then remaining in solution precipitated by dialyzing its

solution into large quantities of alcohol. By dissolving this precipitate in distilled water, and again dialyzing its clear solution into alcohol, the preparation here called "protease" was obtained. The "protoprotease" was made by dissolving the "protease" in ten times its weight of water, saturating the solution with sodium chlorid, and adding dilute acetic acid, saturated with sodium chlorid, as long as a precipitate was formed. The resulting precipitate was then washed with a saturated sodium chlorid solution, dissolved in water, and the solution dialyzed in water until free from chlorin. By then dialyzing the solution into alcohol the "protoprotease" was precipitated, and after being washed with absolute alcohol and dried over sulphuric acid it yielded the preparation used for these experiments.

The results here recorded are of interest as they show it to be possible to separate several proteins from each other, even when present in the same extract. They also show that the products thus

TABLE 9
"PROTEOSE," LENTIL, ERVUM LENS

Experiment	Sensitizing Dose	Second Dose	Result	Protection Test
1	0.005 gm. proteose, lentil	0.05 gm. proteose, lentil	Severe.....	Complete
2	0.005 gm. proteose, lentil	0.05 gm. proteose, lentil	Died, 1 hr.	
3	0.005 gm. proteose, lentil	0.05 gm. eorylin, hazel-nut	0	None
4	0.005 gm. proteose, lentil	0.05 gm. corylin, hazel-nut	0	None
5	0.001 gm. proteose, lentil	0.05 gm. legumin, lentil	0	None
6	0.001 gm. proteose, lentil	0.05 gm. legumin, lentil	0	None
7	0.001 gm. proteose, lentil	0.05 gm. legumin, lentil	0	None
8	0.001 gm. proteose, lentil	0.05 gm. legumin, lentil	0	None
9	0.001 gm. legumin, lentil	0.05 gm. proteose, lentil	0	None
10	0.001 gm. legumin, lentil	0.05 gm. proteose, lentil	0	None
11	0.001 gm. proteose, soy-bean	0.05 gm. proteose, lentil	Doubtful...	None
12	0.001 gm. proteose, soy-bean	0.05 gm. proteose, lentil	Doubtful...	None
13	0.005 gm. proteose, kidney-bean	0.05 gm. proteose, lentil	Slight.....	None
14	0.005 gm. proteose, kidney-bean	0.05 gm. proteose, lentil	Slight.....	None

separated are chemically distinct from one another. According to previous investigations the pea contains two globulins, legumin and vicilin; an albumin, legumelin; and one or more "proteoses." The reactions given in Table 8 show that the "protease" not only has been very completely separated from legumin and vicilin, but that it is a chemically distinct protein. The reactions between the preparation designated "protoprotease" and that designated "protease" give no evidence of more than one substance in the total "protease."

The preparation of "proteose" from the lentil used for these experiments was made in the same way as the "proteose" preparation from the pea (see under Table 8).

The results show that the separation of legumin and "proteose" was very complete, and that the latter is a chemically distinct protein. Lentil proteose is also quite distinct from the "proteose" of the soy bean (*Glycine hispida*), and the proteose of the kidney bean (*Phaseo-*

TABLE 10
"PROTEOSE," SOY-BEAN, *GLYCINE HISPIDA*

Experi- ment	Sensitizing Dose	Second Dose	Result	Protection Test
1	0.01 gm. proteose, soy-bean	0.05 gm. proteose, soy-bean	Died, 15 min.	
2	0.01 gm. proteose, soy-bean	0.05 gm. proteose, soy-bean	Died, 60 min.	
3	0.001 gm. proteose, soy-bean	0.002 gm. proteose, soy-bean	Died, 2 hr.	
4	0.001 gm. proteose, soy-bean	0.001 gm. proteose, soy-bean	Severe	
5	0.001 gm. proteose, soy-bean	0.0005 gm. proteose, soy-bean	Moderate	
6	0.001 gm. proteose, soy-bean	0.0001 gm. proteose, soy-bean	Moderate	
7	0.001 gm. proteose, soy-bean	0.0001 gm. proteose, soy-bean	Moderate	
8	0.001 gm. proteose, soy-bean	0.00005 gm. proteose, soy-bean	Slight	
9	0.001 gm. proteose, soy-bean	0.00001 gm. proteose, soy-bean	Doubtful	
10	0.01 gm. proteose, soy-bean	0.10 gm. glycinin, soy-bean	Slight.....	Partial
11	0.01 gm. proteose, soy-bean	0.10 gm. glycinin, soy-bean	Slight.....	Partial
12	0.01 gm. glycinin, soy-bean	0.05 gm. proteose, soy-bean	Severe.....	Partial
13	0.01 gm. glycinin, soy-bean	0.05 gm. proteose, soy-bean	Moderate...	Partial
14	0.001 gm. proteose, soy-bean	0.05 gm. proteose, lentil	Doubtful...	None
15	0.001 gm. proteose, soy-bean	0.05 gm. proteose, lentil	Doubtful...	None
16	0.001 gm. proteose, soy-bean	0.05 gm. proteose, pea	Moderate...	None
17	0.001 gm. proteose, soy-bean	0.05 gm. proteose, pea	Slight.....	None
18	0.005 gm. proteose, pea	0.05 gm. proteose, soy-bean	Slight.....	None

lus vulgaris). (This preparation of "proteose" from the kidney bean was too toxic for normal guinea-pigs, which it killed in eight to sixteen hours in 0.05 gm. doses, to be used for anaphylaxis experiments except in small doses for sensitization.¹⁴)

From the foregoing data it is plain that the soy-bean "proteose" is chemically distinct from glycinin and exhibits strong anaphylacto-

genic properties. The preparation of proteose appears to be free from glycinin, but the glycinin contains enough proteose to sensitize, altho only enough to produce slight intoxication.

Experiments 14, 15, and 16 show that the preparation of globulin was free from "proteose," and, in conjunction with Experiments 1

TABLE 11
"PROTEOSE," ADZUKI-BEAN, PHASEOLUS RADIATUS

Experi- ment	Sensitizing Dose	Second Dose	Result	Protection Test
1	0.01 gm. proteose, adzuki-bean	0.05 gm. proteose, adzuki-bean	Died, 45 min.	
2	0.001 gm. proteose, adzuki-bean	0.001 gm. proteose, adzuki-bean	Died, 55 min.	
3	0.001 gm. proteose, adzuki-bean	0.0005 gm. proteose, adzuki-bean	Slight	
4	0.001 gm. proteose, adzuki-bean	0.0002 gm. proteose, adzuki-bean	Slight	
5	0.001 gm. proteose, adzuki-bean	0.0001 gm. proteose, adzuki-bean	Doubtful	
6	0.001 gm. proteose, adzuki-bean	0.05 gm. proteose, lentil	0	Partial; moderate reaction
7	0.001 gm. proteose, adzuki-bean	0.05 gm. proteose, lentil	0	Partial; moderate reaction
8	0.001 gm. proteose, adzuki-bean	0.05 gm. proteose, pea	0	Partial; moderate reaction
9	0.001 gm. proteose, adzuki-bean	0.05 gm. proteose, pea	0	Partial; moderate reaction
10	0.01 gm. proteose, adzuki-bean	0.05 gm. globulin, adzuki-bean	Slight.....	Partial; moderate reaction
11	0.01 gm. proteose, adzuki-bean	0.05 gm. globulin, adzuki-bean	Died, 30 min.	
12	0.01 gm. proteose, adzuki-bean	0.05 gm. globulin, adzuki-bean	Moderate....	Partial; moderate reaction
13	0.01 gm. globulin, adzuki-bean	0.05 gm. globulin, adzuki-bean	Died, 20 min.	
14	0.01 gm. globulin, adzuki-bean	0.05 gm. proteose, adzuki-bean	0	Partial; moderate reaction
15	0.01 gm. globulin, adzuki-bean	0.05 gm. proteose, adzuki-bean	0	Partial; moderate reaction
16	0.01 gm. globulin, adzuki-bean	0.05 gm. proteose, adzuki-bean	Slight.....	Partial; moderate reaction

and 13, that these are distinctly different substances. Experiments 10, 11, and 12 indicate that this preparation of "proteose" contained enough globulin to sensitize the animals strongly.

Having found that the so-called proteose obtained from extracts of the hemp-seed has high anaphylactogenic power, we have tested the artificial proteoses made from edestin by hydrolyzing with 2 percent sulphuric acid, and also by heating with water under pressure. We have also tested the anaphylactogenic power of a preparation of proteose made by hydrolyzing zein with 2 percent sulphuric acid. For these valuable preparations we are indebted to Prof. F. P. Underhill. The outcome of these experiments is given in the following tables.

The slight reaction shown by animals sensitized with 0.01 gm. of these artificial proteoses when injected with 0.10 gm. of the same, can as well be ascribed to an incomplete separation of edestin from the preparation used as to feeble anaphylactogenic power of these artificial proteoses themselves. This assumption is made probable by other experiments which strongly indicate the presence of enough edestin in the preparations of proteose highly to sensitize the animals to edestin

TABLE 12
ARTIFICIAL PROTEOSE, MADE FROM EDESTIN BY HYDROLYSIS WITH 2 PERCENT H_2SO_4

Experiment	Sensitizing Dose	Second Dose	Result	Protection Test
1	0.01 gm. proteose, edestin	0.10 gm. proteose, edestin	Slight.....	Still reacts to edestin
2	0.01 gm. proteose, edestin	0.10 gm. proteose, edestin	Slight.....	Still reacts to edestin
3	0.01 gm. proteose, edestin	0.10 gm. edestin, hemp-seed	Died, 60 min.	
4	0.01 gm. proteose, edestin	0.10 gm. edestin, hemp-seed	Severe.....	Complete. No reaction to edestin
5	0.01 gm. proteose, edestin	0.05 gm. proteose, hemp-seed	0	No reaction to edestin proteose
6	0.01 gm. proteose, edestin	0.05 gm. proteose, hemp-seed	0	No reaction to edestin proteose
7	0.01 gm. proteose, edestin	0.10 gm. proteose, hemp-seed	Doubtful...	Severe reaction to edestin
8	0.01 gm. proteose, edestin	0.10 gm. proteose, hemp-seed	0	Severe reaction to edestin
9	0.01 gm. edestin, hemp-seed	0.10 gm. proteose, edestin	Slight.....	Slight reaction to edestin
10	0.01 gm. edestin, hemp-seed	0.10 gm. proteose, edestin	Slight.....	Slight reaction to edestin

ARTIFICIAL PROTEOSE MADE FROM EDESTIN BY HYDROLYSIS WITH H_2O IN AUTOCLAVE

11	0.01 gm. proteose, edestin	0.10 gm. proteose, edestin	Slight.....	Still reacts to edestin
12	0.01 gm. proteose, edestin	0.10 gm. proteose, edestin	Slight.....	Still reacts to edestin
13	0.01 gm. proteose, edestin	0.10 gm. proteose, hemp-seed	0	Still reacts to edestin
14	0.01 gm. proteose, edestin	0.10 gm. proteose, hemp-seed	0	Still reacts to edestin
15	0.01 gm. proteose, edestin	0.10 gm. edestin, hemp-seed	Doubtful...	Does not react to edestin
16	0.01 gm. proteose, edestin	0.10 gm. edestin, hemp-seed	Doubtful...	Does not react to edestin
17	0.01 gm. proteose, edestin	0.10 gm. edestin, hemp-seed	Severe	
18	0.01 gm. proteose, edestin	0.10 gm. edestin, hemp-seed	Died	

(Experiments 3, 4, 17, and 18), but not enough to intoxicate animals sensitized with edestin (Experiments 9 and 10). Experiments 5, 6, 7, 8, 13, and 14 show that the natural proteose of the hemp-seed is chemically distinct from these artificial derivatives of edestin.

This artificial zein proteose, made by acid hydrolysis of zein, is entirely inert, both as regards sensitizing and intoxicating properties, in contrast with the natural "proteoses" of seeds.

Two preparations of artificial zeose made by B. M. Hendrix were also tested, and found not to cause any reactions whatever, or to sensitize to zein. These proteoses were prepared as follows:

The zein was suspended in 0.2 percent hydrochloric acid and some Parke-Davis pepsin added to the solution; the mixture was placed in an incubator at 37 C. and allowed to digest for two days. It was then filtered and the insoluble material was treated as before for two days. This was continued until practically all went into solution. The filtrate was neutralized and filtered and then saturated with ammonium sulphate. The zeose which was thus precipitated was then dissolved in a small quantity of water, dialyzed until free from ammonium sulphate, and its solution evaporated to dryness.

Since both zein and edestin, the proteins from which our artificial proteoses were prepared, are relatively inactive in anaphylactic reactions,¹⁵ proteoses were prepared from squash seed globulin (for this

TABLE 13
ARTIFICIAL PROTEOSE MADE FROM ZEIN BY HYDROLYSIS WITH H₂SO₄

Experiment	Sensitizing Dose	Second Dose	Result
1	0.01 gm. proteose, zein.....	0.10 gm. proteose, zein.....	None
2	0.01 gm. proteose, zein.....	0.05 gm. proteose, zein.....	None
3	0.01 gm. proteose, zein.....	0.05 gm. proteose, zein.....	None
4	0.01 gm. proteose, zein.....	0.05 gm. zein.....	None
5	0.01 gm. proteose, zein.....	0.05 gm. zein.....	None
6	0.01 gm. proteose, zein.....	0.05 gm. zein.....	None
7	0.01 gm. proteose, zein.....	0.05 gm. zein.....	None
8	0.01 gm. zein.....	0.10 gm. proteose, zein.....	None

preparation of globulin we are indebted to Dr. Isaac F. Harris), which is one of the most active anaphylactogens of all the vegetable proteins that we have studied.

The proteoses were prepared by digesting with pepsin-HCl until but a small amount of globulin could be precipitated by one-third saturation with ammonium sulphate. After removal of the globulin, two preparations of proteose were made, one containing all the material precipitated between half saturation and full saturation, therefore consisting chiefly of "deutero" proteoses (Preparation 1), and the other containing the precipitate coming down between one-third and full saturation, therefore presumably containing both "primary" and "deutero" proteoses (Preparation 2).

The results of experiments with these preparations were as follows:

According to the results of these experiments, it would seem that our Preparation 1 of proteose from the squash-seed globulin contains traces of globulin sufficient to sensitize to the latter, but not sufficient

to intoxicate sensitized guinea-pigs. Proteose 1 itself causes only slight effects when given in doses of 50 milligrams to sensitized animals. It is thus evident that this typical proteose, prepared by peptic digestion, is entirely different from the so-called proteoses found preformed in seeds. Proteose 2 shows more activity than Proteose 1, which contains the fraction precipitated between half and full satura-

TABLE 14
ARTIFICIAL PROTEOSE MADE FROM SQUASH-SEED GLOBULIN BY PEPSIN

Experiment	Sensitizing Dose	Second Dose	Result	Reaction Test
1	0.003 gm. Proteose 1, squash-seed globulin	0.05 gm. Proteose 1, squash-seed globulin	Slight	
2	0.003 gm. Proteose 1, squash-seed globulin	0.05 gm. Proteose 1, squash-seed globulin	Moderate	
3	0.003 gm. Proteose 1, squash-seed globulin	0.05 gm. globulin, squash-seed	Died, 1 hr. 40 min.	
4	0.003 gm. Proteose 1, squash-seed globulin	0.05 gm. globulin, squash-seed	Moderate	
5	0.003 gm. globulin, squash-seed	0.05 gm. Proteose 1, squash-seed globulin	0	Partial
6	0.003 gm. globulin, squash-seed	0.05 gm. Proteose 1, squash-seed globulin	0	Partial
7	0.005 gm. Proteose 2, squash-seed globulin	0.05 gm. Proteose 2, squash-seed globulin	Moderate	
8	0.005 gm. Proteose 2, squash-seed globulin	0.05 gm. Proteose 2, squash-seed globulin	Moderate	
9	0.005 gm. Proteose 2, squash-seed globulin	0.05 gm. Proteose 2, squash-seed globulin	Doubtful	
10	0.005 gm. Proteose 2, squash-seed globulin	0.05 gm. Proteose 2, squash-seed globulin	Doubtful	
11	0.005 gm. Proteose 2, squash-seed globulin	0.05 gm. Proteose 2, squash-seed globulin	Died, 95 min.	
12	0.005 gm. Proteose 2, squash-seed globulin	0.05 gm. globulin, squash-seed	Died, 50 min.	
13	0.005 gm. Proteose 2, squash-seed globulin	0.05 gm. globulin, squash-seed	Died, 40 min.	
14	0.005 gm. Proteose 2, squash-seed globulin	0.05 gm. globulin, squash-seed	Died, 40 min.	
15	0.005 gm. Proteose 2, squash-seed globulin	0.05 gm. globulin, squash-seed	Severe.....	Complete
16	0.005 gm. globulin, squash-seed	0.05 gm. Proteose 2, squash-seed globulin	Died, 25 min.	
17	0.005 gm. globulin, squash-seed	0.05 gm. Proteose 2, squash-seed globulin	Died, 25 min.	
18	0.005 gm. globulin, squash-seed	0.05 gm. Proteose 2, squash-seed globulin	Moderate...	Partial
19	0.005 gm. globulin, squash-seed	0.05 gm. Proteose 2, squash-seed globulin	Moderate...	Partial

tion with $(\text{NH}_4)_2\text{SO}_4$, possibly because it contains sufficient unaltered globulin to produce some reaction, even when used as the intoxicating dose.

SUMMARY

The reaction of anaphylaxis demonstrates that those vegetable protein preparations, commonly designated "proteoses" because their solubility is like that of the proteoses produced by the action of pepsin or trypsin on native proteins, are distinguishable by biologic reactions and

therefore are chemically distinct from the other reserve proteins of the seeds. The "proteoses" obtained from different seeds and grains are also quite distinct from one another.

They exhibit strong anaphylactogenic properties and cause very severe anaphylactic intoxication when injected into sensitized guinea-pigs, even in minute doses (.001 to .0005 gm. being fatal doses with some of them). Their activity in this respect resembles that of the soluble animal proteins, such as egg-white, serum, etc., and greatly exceeds that of any of the other vegetable proteins (globulins, alcohol-soluble proteins, etc.) which we have studied. This high anaphylactic activity seems to depend upon their great solubility in the body fluids, as shown in Paper V of this series.¹⁶ Their activity is not destroyed by heating at 100 C. for one-half hour, presumably because of their incoagulability.

In their anaphylactic power these "natural proteoses" differ sharply from proteoses obtained from animal proteins by digestion with enzymes, or by chemical hydrolysis, such artificial products being almost, if not entirely, non-anaphylactogenic. Furthermore, those products of hydrolysis which result from heating vegetable proteins with acids, with water under pressure, or by peptic digestion, have, so far as we have tested them, no anaphylactogenic properties. From these facts it would seem that the vegetable "proteoses" belong to a group of proteins which are chemically different from any heretofore recognized. They resemble highly soluble native proteins in their anaphylactogenic capacity, and are probably quite as complex in their chemical constitution. Their designation as "proteoses" is consequently improper.

This differentiation of so many of these "protease" preparations from the other proteins in the same seed is a striking example of the fact that specificity of the anaphylaxis reaction is not dependent on biologic origin, but on chemical constitution. Our experiments show also that these soluble proteins, which occur in the seeds of so many species of plants, are as distinct from one another as are the other more abundant proteins.

The results described in this paper demonstrate that anaphylaxis furnishes a useful means for determining the purity of protein preparations obtained from plant extracts, in so far as contamination with the other proteins of the same seed is concerned.

16. Jour. Infect. Dis., 1914, 14, p. 377.

The Journal of Infectious Diseases

PUBLISHED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 17

September, 1915

No. 2

ENVIRONMENTAL STUDIES OF STREPTOCOCCI WITH SPECIAL REFERENCE TO THE FERMENTATIVE REACTIONS *

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Four recent articles on streptococci by Winslow, Thro, Lyall, and Hopkins and Lang have included most of the historical review originally a part of this paper. Additions to these historical surveys will be found in Table 17, or in the foot-note accompanying it.

The investigations just mentioned cover five different phases: (1) the qualitative determination of acid and the correlation of the results in the various media (as used by Gordon, Andrewes and Horder, and Houston); (2) the constancy of the reactions of streptococci, especially in the various media originally suggested by Gordon (tested quite incompletely by most investigators); (3) the application of the biometric method introduced by Andrewes and Horder; (4) the substitution of quantitative for the original qualitative measures, or standards, in the biometric applications, as emphasized chiefly by Winslow and his associates; and (5) the correlation of the reactions of streptococci with their habitats in an attempt to gain results of "practical sanitary importance."

* Received for publication May 4, 1915. Part of the investigation was prosecuted with the aid of a grant from the Esther Herrman Research Fund of the New York Academy of Sciences.

This paper gives a condensed account of the results obtained in a study of 767 strains of streptococci, of which all but twenty-three were freshly isolated for this purpose. The account is subdivided as follows:

I. Constancy of the streptococci (measured chiefly by fermentative activities, but also by other characteristics and powers, as, for example, colony characteristics on agar, effects upon blood agar, gelatin, etc.)

(a) As exhibited at varying periods after isolation, and

(b) As affected by variations in environment (1) within the range of possible laboratory variations in media, technic, etc., and (2) more extreme, designed to induce changes in streptococci.

II. Characteristics of the streptococci on isolation from various sources or habitats. These were studied to ascertain whether streptococci bear or retain sufficient impress of a given environment to yield diagnostic differences—differences distinctive enough, for instance, to aid in practical sanitary investigations.

I. CONSTANCY

Any study of the characteristics* of streptococci, morphologic or physiologic, introduces at once the question of constancy. To avoid numerous digressions and repetitions during the following discussion, it will be necessary to consider first this still open question of constancy of the streptococci.

As shown in an earlier paper, constancy may be viewed in two ways: (1) Constancy may be used for the sum total of identical responses exhibited by a given strain or strains under duplicate or identical conditions (necessarily contemporary); or, (2) constancy may be broadened to include the power of continuing such identical responses under varying or varied conditions. And, fairly or not, that is what most of us demand of a given strain before we term it "constant." Constancy throughout a given period falls under this second type of constancy; for with the greatest possible care no one can insure complete uniformity of all conditions during a period of several months, or even weeks. This is primarily because even the media we use are substances still too completely unknown, and therefore factors unknown; there are, of course, other unknown influences, including the continued effects of bacterial products upon the bacteria themselves.

* Details concerning the preparation of media, titration methods, stains, and other matters of technic are all placed together in the appendix.

Constancy of the first type—under identical conditions—has been vouched for by Hilliard. From about 240 strains of streptococci, he made duplicate inoculations into saccharose, lactose, etc., securing three days later similar titration results for practically all of the 240 pairs in five or six media each. In my own work, several hundred such duplicate strains were tested; a variation

TABLE 1
REGIONAL LIKENESSES OF STREPTOCOCCI

Strains from Cat 3	Region	Saccharose	Lactose	Salicin	Raffinose	Mannite	Inulin
44	Cardiac, stomach, contents	0.1	2.7	5.1	0.0	3.5	0.2
45	Cardiac, stomach, contents	0.2	3.4	5.1	—0.1	3.5	0.2
46	Cardiac, stomach, contents	0.2	1.2	4.9	0.0	3.6	0.3
47	Cardiac, stomach, contents	5.2	4.4	4.3	1.7	0.1	0.1
48	Cardiac, stomach, contents	0.2	2.2	4.8	0.0	3.6	0.3
49	Cardiac, stomach, contents	0.3	2.6	5.0	0.0	3.6	0.2
50	Cardiac, stomach, contents	0.2	2.6	5.0	0.0	3.8	0.3
51	Cardiac, stomach, contents	0.2	2.3	4.9	0.0	—0.2	0.1
54	Mucous membrane duodenum	0.2	2.5	4.8	0.1	3.6	0.1
55	Mucous membrane duodenum	0.0	2.3	5.3	0.0	3.4	0.2
56	Mucous membrane duodenum	0.2	3.1	3.7	0.1	4.1	0.1
57	Mucous membrane duodenum	0.0	2.6	6.0	—0.1	3.6	0.2
58	Mucous membrane duodenum	0.3	2.3	5.0	0.0	3.4	0.2
59	Mucous membrane duodenum	0.1	2.0	4.7	0.0	3.3	0.4
60	Mucous membrane duodenum	0.1	2.4	5.4	0.0	3.7	0.1
61	Mucous membrane duodenum	0.2	1.5	4.8	0.1	3.6	0.2
62	Small intestine, contents	0.0	1.7	5.2	0.2	3.5	0.2
63	Small intestine, contents	0.3	1.8	4.9	0.0	3.4	0.1
104	Small intestine, contents	0.2	2.3	4.5	0.1	2.8	0.2
101	Mucous membrane, small intestine	0.2	2.2	4.5	0.2	3.2	0.2
52	Large intestine, contents	0.1	2.5	5.7	0.0	3.6	0.2
53	Large intestine, contents	0.1	2.1	5.0	0.0	3.7	0.1
102	Large intestine, contents	0.2	1.6	4.5	—0.1	2.7	0.2
103	Mucous membrane cecum	4.4	0.2	3.7	0.7	0.1	1.1

in acidity of more than 0.2 or 0.3 occurred in less than 0.5 percent of the tubes titrated. (Differences greater than this were usually found only in (1) mutating (?) strains, in which a new fermenting power was suddenly acquired, evincing itself in one tube more markedly than in another; or (2) in weak strains that were dying out. Repetition in the first case invariably yielded like positive results. These exceptions were but few in number, and relatively

unimportant.) The same parallelism was, as might be expected, exhibited by these duplicate strains in other media not quantitatively measured: litmus milk, broth of various kinds, blood agar, gelatin, etc.

This doubtless explains the marked similarity often shown by strains from the same sample; for example, Winslow and Palmer (1910). This similarity characterized strains isolated from the same sample in my own work, even though an effort was made to select strains showing morphologic differences (in size, chain length, regularity in the plane of cell division, relative frequency of abnormal cells in the chain, etc.); differences in the characters on agar and in broth were also considered in selecting the strains. The same detailed likenesses were often presented not only by the strains from several samples from a given locality, such as the samples from various parts of the stomach or of the intestine, but by those from different regions of one individual—stomach, small intestine, cecum, and large intestine (see Table 1). Because of this similarity, usually but one to three strains were taken from each sample.

TABLE 2
CONTEMPORARY FLUCTUATIONS IN TWO SUBCULTURES OF ONE STRAIN OF STREPTOCOCCUS

Subcultures	Date of Test	Saccharose	Lactose	Salicin	Raffinose	Mannite	Inulin
98a	January 23	2.8	2.5	2.3	0.2	2.0	2.7
98b	January 23	2.7	2.3	1.7	0.0	1.7	2.7
98a	February 25	3.7	2.4	2.1	2.4	2.0	0.0
98b	February 25	2.8	2.6	2.0	2.4	2.1	0.0
98a	April 10	3.4	3.0	1.9	2.6	2.5	2.6
98b	April 10	3.9	3.1	2.4	3.1	2.9	2.6
98a	May 16	3.3	3.1	2.3	2.8	2.5	2.8
98b	May 16	3.0	3.3	2.5	2.6	2.6	2.6

Unless some such scheme of limiting the number from each sample is followed, as readily will be seen, one may draw entirely erroneous conclusions regarding the relative frequency of the various types of reactions in a given locality or habitat. For example, a disproportionate number of strains from Cat 3 (Table 1), in which twenty-one of twenty-four strains fermented mannite, would lead to an erroneous view concerning the relative frequency of mannite fermenters in the alimentary canal of cats; for 87 percent of the strains from this cat fermented mannite, but in the four other cats studied (a total of eighty-nine strains) only 35 percent fermented mannite.

This identity of response shown in freshly isolated strains often continues to manifest itself in duplicate strains carried along independently for a given time. This is illustrated in Table 2, by the similar, contemporary fluctuations in two subcultures of Strain 98 (cat, palate). Other strains might be added confirming by such contemporary fluctuations the conclusion that a given strain at a given time under identical conditions shows an identity of response in the sum total of reactions. Such parallel fluctuations are strong arguments for constancy of the first type.

But, as just shown, these responses may vary with age. Changes due to age or to more or less favorable conditions for growth belong under the discussion of constancy of the second type; that is, constancy under varying conditions, more or less definitely known.

CONSTANCY IN RELATION TO AGE

In any laboratory the period necessary for isolation is variable, especially when feces and similar materials are used as sources. And, if one is working "to capacity," the routine can never be so well established that it is possible to use only cultures that (1) fall within a brief, fixed isolation period and (2) are fresh transfers, eighteen to twenty-four hours old. One often has to choose between making fresh transfers or using slants more than twenty-four hours old.

To see the possible effects of such differences, slants (of about twenty strains) were compared with their respective subcultures, both being inoculated into the test media at the same time; the results obtained from twenty-four-hour slants were also compared with those obtained from the same slants when they were five to eight days old.

Even in the fermentative reactions the differences were negligible. While such variations in laboratory routine might make a difference with less hardy strains, allowing them to die off before the special or test media were inoculated, one apparently need not expect any wide range in the final results that could be attributed to age differences of one to three days, which is as wide a range as would be found in most laboratories.

This second view of constancy (under varying conditions) was still further tested for the effects of age on the fermentative activities: A number of strains (twenty) were kept on slants at room temperature for one year. Four survived; they were repeated in the usual test media with the results shown in Table 3. As is apparent, they were remarkably constant* in view of the uncongenial conditions to which they were subjected; Strains 47 and 93 had both lost the power of fermenting raffinose. In this connection, it is interesting to know that Strain 93 did not, when first isolated, ferment raffinose, but gained that power suddenly during the sixth month, just before the slant for drying was prepared. As will be shown in later studies, the fermenting power latest in development or manifestation is the one most easily lost.

It was not, of course, expected that the results with these four strains would yield anything of more than passing interest. It is much more important to know how constant strains are under ordinary laboratory conditions—whether we can compare strains freshly isolated with older ones loaned by other laboratories, etc.

With this in mind, 134 cultures, representative of the various types of bacteria, were selected as they were isolated and kept as stock cultures to be tested as often as opportunity allowed. Such strains were transferred every ten days, incubated twenty-four hours at 37 C., and stored (the remaining nine days) at room temperature in closed containers to prevent drying out. At intervals of two weeks, four weeks, two to four months, six months, and twelve months or longer, they were again tested in the usual Gordon media. Strains proving inconstant were usually dropped, tho a number of inconstants were carried on to note the trend of the changing reactions.

* Complete constancy of this kind has been reported by Holman (1914) for one strain of *Streptococcus faecalis*, dried on a cover glass for over five months. All of my survivors were short-chained streptococci.

TABLE 3
COMPARATIVE RECORDS AFTER DRYING STREPTOCOCCI FOR ONE YEAR

Strain	Source	Time Since Isolation	Longest Chain Length in any Media	Order of Frequency	Clearing in Broth	Agar Colonies	Gelatin	Litmus Milk Time of Coagulation	Color in 10 Days	Blood Agar	Saccharose	Lactose	Salt solution	Raffinose	Manite	Indulin
47	Oat 3, stomach	6 mo. 1 year later	10 6	4.2 2.4	None 10 da.	Usual Slight muggy	2 da. 18 hr.	3 da. 3 da.	Top half pink Nearly all pink	Greenish Pale greenish	3.8 4.3	3.3 3.3	3.4 3.3	1.3 0.5	0.0 0.2	0.0 0.1
93	Oat 5, stomach	6 mo.	6	2.4	5 da.	Usual to slight, heavier Slight muggy	24 hr. 18 hr.	None Slight	Pink Pink	Greenish Greenish to no color	3.9 4.2	2.5 3.8	3.1 3.3	1.8 0.5	0.1 0.0	0.0 0.2
165	Dog 10, throat	2 da. 1 year later	6 6	2.4 2.4	None None	Muggy Muggy	24-hr. liquefaction 24-hr. liquefaction	2 to 3 da. 2 to 3 da.	Top half pink; side H ₂ O Top half pink	Greenish Greenish	3.1 3.6	2.9 3.9	3.6 4.5	0.1 0.3	2.6 3.3	0.1 0.2
293	Dog 5, small intestine	1 da. 1 year later	12 6	4.2 2.4	10 da. 5 da.	Usual to muggy Usual	24 hr. 2 da.	None None	Pink Pink	Greenish to hazy No color; slightly hazy	0.1 0.2	3.3 3.3	4.5 4.3	-0.1 0.0	0.0 -0.1	0.0 0.2

The 134 strains tested for constancy were from the sources listed below, each strain (except the water strains) being isolated from a different sample.

Unknown (from other laboratories).....	5
Hay and water.....	4
Milk	14
Blood, abscesses, etc.....	19
Normal animals:	
Cats (a) throat	9
(b) alimentary canal	13
(c) feces	2
Dogs (a) throat	8
(b) alimentary canal	9
(c) feces	14
(d) recovered in feeding experiments.....	12
Man (a) throat	7
(b) feces	11
Horse, feces	4
Hen, alimentary canal.....	3
Total	134

These repetitions are discussed in two groups: (1) Successive titration records for 134 strains in six Gordon media, at intervals as just described; (2) more complete records, including milk, gelatin and agar plates, blood, etc., as well as the Gordon media, for thirty-one of these strains compared after at least one year on artificial media. The 134 strains are analyzed first with regard to constancy in their fermenting powers in the six Gordon media used (saccharose, lactose, salicin, raffinose, mannite, and inulin).

All strains tested for constancy were kept on plain meat agar, so that the gain in any given case does not mean learning to use a substance to which it had been subjected. That will be discussed in a later section. While some inconstant strains were dropped now and then, constant strains also suffered the same fate, the aim being to observe as many different types as possible (morphologic, as well as physiologic); and similar and apparently fixed types were often dropped to make room or time for new ones, particularly for those not typical of the locality from which they were secured. That this procedure was not selective in the sense of influencing the percentages of constancy, is indicated by comparing the constants for the three-month, and for the four-month period. Only two strains were retested in both the three-month and the four-month lots. The three-month retests gave 63 percent constant, while the four-month retests gave 64 percent constant.

This is probably due to the fact that each strain was more constant than variable; for example, in Strain 81 (from the throat of a cat) we

have, as shown in Table 5, but four changes of a possible total of thirty, or a constancy of 86 percent.

As this table shows, the percentage of constants is about the same at any period during the first year. Or, to state it another way, the 134 strains retested at intervals averaged three retests each. In all, 345 retests were made, including duplicate and intermediate retests

TABLE 4
RESULTS OF TESTS FOR CONSTANCY IN STRAINS OF STREPTOCOCCI WITH RELATION
TO AGE

Number of Retests	Time Since First Test	Strains Constant
26	11—17 da.	20 (76%)
29	1 mo.	20 (68%)
43	2 mo.	33 (76%)
19	3 mo.	12 (63%)
48	4 mo.	31 (64%)
57	6-7 mo.	42 (73%)
27	12-13 mo.	21 (77%)
6	15 mo.	4 —
3	21-24 mo.	1 —
Total 258		

not given in Table 4. In 262 retests, the results in all six of the Gordon media were like those of the latest tests; in 83 retests, changes occurred, usually in but one, sometimes in two, substances, giving really a constancy of over 93 percent.

My percentage of constants was higher than the percentages obtained by several workers, and lower than those given by others.

TABLE 5
RECORD OF ONE STRAIN OF STREPTOCOCCI FOR SIX MONTHS

	Saccharose	Lactose	Salicin	Raffinose	Mannite	Inulin
January 21...	+	+	+	+	—	+
February 25...	+	+	+	+	—	+
April 1.....	+	+	+	—	—	—
April 15.....	+	+	+	—	—	—
May 16.....	+	+	+	+	—	+
July 21.....	+	+	+	+	—	+

Those who have tested the greater number of strains (e. g., Lyall) report more favorably regarding constancy. The percentage of constants is, of course, directly related to the number of test substances used, and, as shown later, affected by the kind of media used, and by the methods of measuring acidity.

In considering constancy several questions arise:

(1) *In which substances are streptococci least variable?*

This can be tested only in those strains which have previously exhibited any designated power. On comparing the repetitions of the 134 strains tested for constancy, we find that, on retesting, the changes or losses are in the following order: in salicin 2 percent; lactose 2 percent; saccharose 4 percent; raffinose 6 percent; mannite 10 percent; and inulin 21 percent.

(2) *Has the relative constancy in various substances any relation to the readiness with which these substances are fermented?*

This relative constancy corresponds in a general way to the order of fermentability of these 134 strains, for 97 percent fermented salicin; 96 percent lactose; 84 percent saccharose; 57 percent mannite; 28 percent raffinose; and 20 percent fermented inulin. It also corresponds to the order of fermentability for the whole 767 strains studied; 88 percent fermented lactose; 85 percent salicin; 74 percent saccharose; 46 percent mannite; 30 percent raffinose; and 24 percent inulin.

(3) *Are newly acquired powers as constant as those of earlier manifestation?*

Newly acquired powers seem more variable. For example, Strain 11 (originally fermenting none of the Gordon media) suddenly began to ferment salicin; during the next five months it lost this power twice, regaining it each time. Other non-fermenting strains have wavered similarly after acquiring salicin. Other strains, originally fermenting one or more substances, show the same fluctuations with their latest acquired powers; mannite or raffinose, or raffinose and inulin. Arkwright (1913), who attributes variation to a general lowering of functional activity, suggests that the power suppressed is probably more recently acquired.

(4) *Is there, with age, a greater tendency to gain or lose in fermentative power?*

There seems under ordinary laboratory conditions a greater tendency to gain than to lose fermenting powers. Among these 134 strains, forty (29 percent) gained one or more substances, while but twenty-six (19 percent) lost substances. There is also this decided difference: A strain that loses a power may, and usually does, regain that power later. But a strain that gains a power usually keeps it; in this the fluctuation is strikingly less than that which attends the loss of a power. Lyall (1914) reports for his 263 pathologic strains

changes by addition only, stating that variants by loss were not observed. Floyd states (in a letter of 1915) that the gains in the Floyd and Wolbach series outnumber the losses; their strains, it will be recalled, contained an unusually high percentage of non-fermenters.

Differences might be expected not only in the number of substances fermented, but in the amount of acid formed. Since strains from the alimentary canal and from strictly non-parasitic sources generally yield a higher percentage of acid, one might argue perhaps that a long period on artificial media might be expected to have such an influence; but apparently age does not affect markedly the titration results. Of these 134 strains, eighty-four (62 percent) did show a gain in the amount of acid formed in a total of 201 substances (averaging $2\frac{1}{2}$ substances a strain); but these strains usually had other records lower than the original ones, and the gains were not at all continuous or progressive.

(5) *Is there any relation between constancy and the source, or origin, of the strains?*

The strains from hay, water, and milk (non-parasitic sources) seem a little less variable than most of the strains (73 percent constant). They are however too few in number to be convincing. The twelve strains from the alimentary canals of dogs after feeding of streptococci are remarkably constant (91 percent); only one strain showed any change in the year through which they were studied. The twenty-three strains isolated from the alimentary canals and the feces of normal dogs are also relatively constant (78 percent), but this may be a mere coincidence. There would seem to be no real reason why strains from dogs should be more constant than those from other sources. (The throat strains from the same dogs were more variable; but throat strains from several other animals studied are apparently both more variable and more varied than those from any other source.)

The general feeling that strains from pathologic conditions are more variable is probably correct, tho Lyall reports less than 5 percent showing variation in his 263 strains, and Floyd and Wolbach but "few" changes in their seventy-five retests. These high proportions constant may be due to the fact that Lyall incubated his tubes for ten days, and Floyd and Wolbach incubated theirs for seven days. My own strains which were recently isolated from such sources would indicate that they are probably the most variable. The number actually tested is so small, however, that no definite statement can be made;

and those obtained from other laboratories were old cultures and usually fermented at least four of the Gordon test substances used in this work.

The variations that occur seem related not only to the number, but to the range of fermentative powers that the strain possesses. This will be included under (6).

(6) *Does the fermentative combination or complex of a strain enable one (a) to forecast with regard to its constancy or (b) to predict the trend of any changes that may occur?*

(a) The highest records for constancy (83 percent) were shown by the strains originally fermenting saccharose, lactose, salicin, and mannite (30 strains). Those originally fermenting saccharose, lactose, salicin, raffinose, and mannite (20 strains) showed 75 percent constant strains. But a tabulated comparison of all the mannite strains showed but 67 percent constant through the periods tested. This is quite within the range of general constancy, so that mannite organisms as such are probably not more constant than other streptococci. (Of the inconstant mannite strains, however, one-fourth varied only in a lapse of the mannite power.) No other prominent group (such as the saccharose-lactose-salicin group) showed more than the usual number of constants.

(b) The substance gained or lost by a given group is apparently in the line of other comparatively stable groups; for example, lactose-salicin strains tend to gain mannite, the lactose-salicin-mannite strains being relatively common, and varying usually in mannite activities only. Saccharose-lactose-salicin strains more often pick up raffinose (12 in 40) than mannite (2 in 40); yet saccharose-lactose-salicin-mannite strains form the largest group constant studied (25 out of 30). It seems to originate in a lactose-salicin group rather than from saccharose-lactose-salicin strains. A strain that begins to ferment raffinose often later (occasionally simultaneously) ferments inulin. (Libman and Celler (1910) observed earlier that the raffinose and inulin fermenting powers are usually associated. Their percent of linkage is evidently a little higher than that shown by my tables in this paper.) On cultivation under ordinary conditions, strains that acquire the mannite power rarely acquire also raffinose or inulin; the reverse of this usually holds also.

The more stable groups are the more common, not only in this series, but in the whole 767 strains studied; for example, lactose-

salicin-mannite strains and saccharose-lactose-salicin-mannite strains (mentioned as stable) are common in feces, the alimentary canal, etc.

In this connection a scheme* has been worked out showing the probable relationship of the various 134 strains. As stated before, several retests were made for each of these strains. Each strain proving constant for the whole period in which it was tested (whether two weeks or two years) is included in the number given in the oblong figures in the accompanying chart. Changes in the fermentative complex causing a strain to be placed in a new group are indicated by the lines connecting the oblong figures. The number of changes between two fermentative complexes is indicated by a figure placed in an arrow. For example, twenty-five saccharose-lactose-salicin-mannite strains remained constant; two others changed to lactose-salicin mannite; eight others changed to saccharose-lactose-salicin-raffinose, mannite; five saccharose-lactose-salicin-raffinose-mannite strains lost raffinose power and fell into the saccharose-lactose-salicin-mannite group, and one lactose-salicin-mannite strain gained saccharose power and entered this group.

With this explanation a glance at Chart 1 will show (1) that these 134 strains included several stable groups of considerable size (e. g. saccharose-lactose-salicin); (2) that between these groups there are usually well-worn paths (e. g., between saccharose-lactose-salicin and saccharose-lactose-salicin-raffinose); (3) that the variation is more often progressive (acquiring rather than losing fermentative powers); and (4) that these combinations fall into three main groups, each probably sharing a different ancestral history, through lactose, through saccharose, or through salicin.

Note, for example, the lower half of the chart, where a probable family-tree of the lactose line is presented. The eighty-eight titrations in this series show that it is possible to pass from a non-fermenter to a lactose fermenter, on to a lactose-salicin fermenter, on to a lactose-salicin-mannite fermenter and so on to a saccharose-lactose-salicin-raffinose-mannite fermenter.

* This scheme includes a record for every change in the titration records of the inconstant strains. In the preceding discussion, an inconstant strain is listed but once; here, however, it is counted each time a change occurs. For example, if a strain changed from lactose-salicin, to lactose-salicin-mannite, and then back to the original lactose-salicin combination, it would be represented in connection with the corresponding arrows twice, once in each direction. No records are included for the times a strain corresponded with its immediately preceding record, the aim being to find the routes traveled in the changes that do occur. Inulin was omitted from this scheme. It followed raffinose closely enough to add little of any value, and the several additional small groups, often of one strain only, complicated the chart and distracted the attention without yielding any apparent compensations.

Saccharose and its additions (65 titration records) lead to the raffinose groups (lacking mannite). A third path of descent through salicin leads less directly either to the mannite or to the raffinose complexes (21 records). The raffinose and mannite complex seems to develop almost entirely from mannite strains with a lactose ancestry.

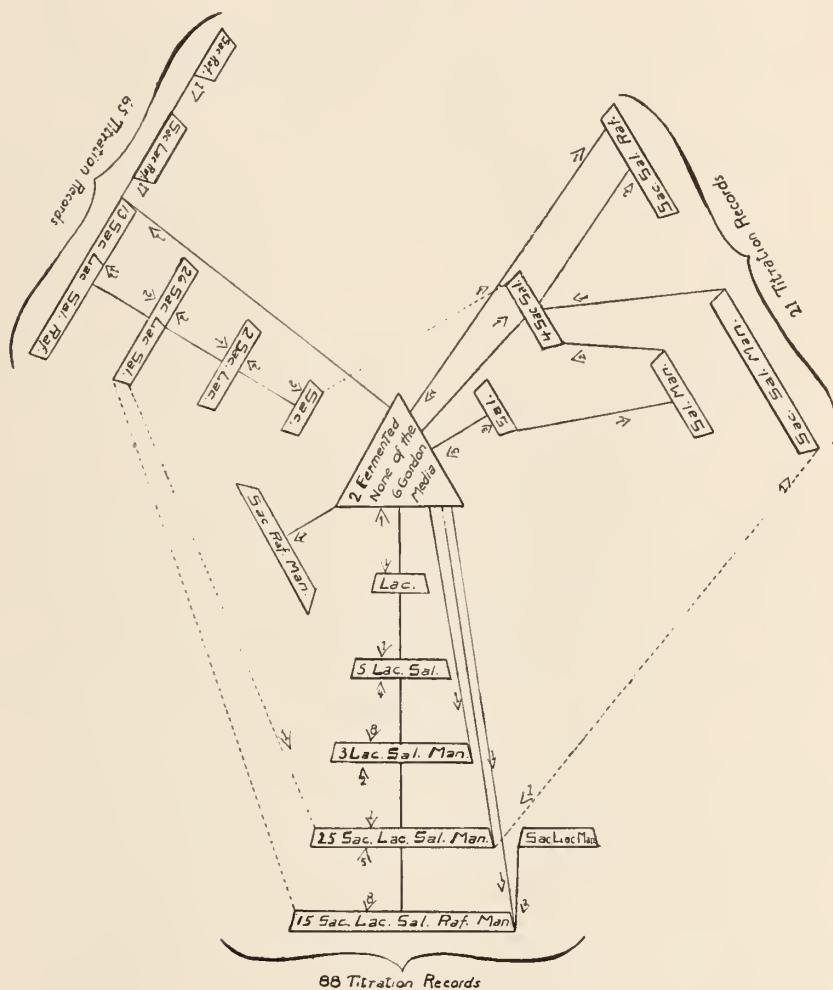


Chart 1.—The possible relationships of the common fermentative complexes. This scheme includes 174 titration records (the repetitions which proved constant are not represented in this chart). Of these, 88 fall into the lactose-mannite line, or group; 65 into the saccharose-raffinose line; and 17 into the salicin (lacking lactose) line.

Note here the small number (4) of cross connections (indicated by dotted lines) between the three "family-tree" lines in Chart 1.

The strains that changed more than once during the period of observation were compared individually with this scheme to see whether they afforded any supporting evidence for this theory of descent. The following facts are of interest:

- (1) Several strains wavered between two well-defined complexes:
 - (a) One strain changed three times from a non-fermenter to a salicin fermenter and back again.
 - (b) Three strains each changed from lactose-salicin to lactose-salicin-mannite and back again.
 - (c) Three strains changed from saccharose-lactose-salicin-mannite to saccharose-lactose-salicin-mannite-raffinose and back again; one strain made the double trip two times.
- (2) Several strains ranged through two or more groups, each keeping nevertheless to the line or series to which this scheme would indicate that it belonged:
 - (a) One strain changed from lactose to lactose-salicin, and later to lactose-salicin-mannite.
 - (b) One strain changed from saccharose-lactose-salicin-mannite to saccharose-salicin-mannite and back to saccharose-salicin.
 - (c) Another strain traveled routes as follows: Saccharose to saccharose-lactose; saccharose-lactose to saccharose-lactose-salicin; saccharose-lactose-salicin to saccharose; saccharose to saccharose-lactose; and saccharose-lactose to saccharose, when the investigation ceased.

The metabolic gradient suggested by Howe (1912) and enlarged upon by Winslow (1912) and Hilliard (1913) may throw some light upon these three divisions or series of streptococci (Chart 1). Raffinose power presupposes the power to use saccharose; out of 234 raffinose fermenters (in the 767 strains studied), but one failed to ferment saccharose (Strain 7, human throat). Mannite similarly includes lactose; out of 360 mannite fermenters, but seven failed to ferment lactose (4 of the 7 fermented salicin, and our chart indicates a possible mannite descent through salicin). Of the strains fermenting both raffinose and mannite, but one lacked the power of using saccharose and lactose (Strain 87, dog feces). The converse of these

metabolic relationships* does not hold, as indicated by the order of fermentability. These relations are interesting as they make plausible, at least, the lines of descent given in Chart 1.

Tho anticipating somewhat a part of a later section of this paper, it seems best to add here the following related comparisons. If this ancestral scheme is biologically sound, it might be expected that the stimulating environments would afford interesting evidence. A comparison of Hilliard's results (1912) with duplicate strains grown at 20 C. and at 37 C. supports this theory. Selecting the strains yielding acid in at least one substance at both 20 C. and 37 C., the only difference we find occurring more than once is a change also represented in our series, saccharose to saccharose-lactose; eight strains fermented saccharose only at 20 C. and saccharose-lactose at 37 C.

The induced changes obtained in my later work also offer some support for these theoretic descents; for example, the change obtained by subjecting Strain 29 to saliva, and also to alimentary canal conditions (collie dog) occurred later in the regular stock culture of 29. It would seem in such cases as if the changed environment provided the needed stimulus, but that the ability to vary and the type of variation are inherent in the organisms.

Changes reported by several other workers fall in with my scheme very well. Most of them, however, used too few strains in the retests or else did not give sufficient details concerning the fermentative reactions to enable me to add to the generalizations already given.

As will be shown later (Table 17), these groups have some habitat affiliations; the strongest is probably shown by my human mouth streptococci, 85 percent of the strains of which fall into the saccharose-raffinose groups; 74 percent of Hilliard's 185 mouth strains are found here, also.

That the variation occurs within the limits shown in this "family-tree" indicates that the most important factor is, after all, the organism itself. Environmental influences may hasten such changes, but the line of change seems to lie with the organism.

Rettger and Sherrick (1911), experimenting with *Bacillus prodigiosus*, state that variation does not depend solely upon changes in nutriment, environmental conditions, etc., but is frequently brought about through inherent proper-

* It is possible that the alcohol group in lactose is definitely related to the power to use mannite, a hexahydric alcohol; the chemical likeness of saccharose and raffinose has also been pointed out by Hilliard (1913). Chamot says (letter of 1915), "There are not a few cases where a bacterial species will ferment certain carbohydrates and also hydrolize some individual glucosid, but so far as I am aware, no one has yet discovered the fundamental reason for this selective action."

ties within the organism itself. And Penfold (1912) states that the "inability of *Bacillus coli* to vary quickly in respect to certain carbohydrates would appear to be as characteristic of certain species as the power to vary." Thro (1914) emphasizes the variability of the organisms, tho he mentions additional causes of variation in the streptococci.

CHANGES WITH AGE OBSERVED IN OTHER MEDIA

Besides the fermentative observations just described for the 134 strains, more general observations were made for thirty-one strains kept for one year (transferred to fresh agar every ten days) and retested in milk, blood, etc., as well as in the Gordon media. The changes are marked (Table 6). It is a surprise to find that the quantitative Gordon results are more constant than the less definitely measurable results in such media as blood, litmus milk, and gelatin. The chain length runs much the same; the characters on agar are relatively constant. Gelatin indicates great differences in the ease with which these strains grow in it at room temperature. (In this connection, recall that these stock strains were kept at room temperature nine of every ten days.) In twelve of fourteen strains, growth in gelatin is earlier than in the freshly isolated strains; in four of these fourteen it is two to ten days earlier. There were no changes with regard to liquefying power.

Broth* is rather disappointing in this connection. Fourteen of twenty-five vary two to five days in the time of clearing. One, clearing on isolation, fails to do so after one year; and one not clearing when isolated clears in five days. There seems to be no distinct tendency to lengthen or shorten the period before clearing. Differences in this regard are often attributed to differences in the reaction of the medium. Forty-eight strains inoculated simultaneously into sugar-free broth and into plain broth of various acidities + 0.5, + 1.5, and - 0.5, and into calcium carbonate plain broth, and 126 strains inoculated into all but the - 0.5 broth indicate that, while there may be a difference of one to two days in the time of clearing, it is apparently due to differences of vigor and amount of growth, due, in turn, probably to the more or less favorable initial acidity. Lyall (1914) finds broth characteristics "so largely dependent on the nature and reaction of the medium" that they seem of little value.

In the various Gordon media, the three-day appearances vary greatly, but the records of 767 strains in the six kinds of media, including many hundred duplicates, totaling about 8,000 tubes, do not yield anything leading to a correlation of turbidity or clearing with the amount of acid formed; nor is there any definite relation, apparently, between the clearing in plain broth and that in the Gordon media. Tho strains clearing in the former usually do so in the latter, the converse is not true, and the effects in sugar broth are decidedly less differential than those in plain broth.

Litmus milk is not much more satisfactory; four of the strains changed with regard to the power of coagulating milk.

Blood probably offers the greatest surprise of all. On blood agar, but few non-green streptococci change to green; ten of the thirty-one strains originally green or with no color changed, forming colonies with a definite hazy area or rim; four gained the power to hemolyze blood; and fourteen (ten of them hemolyzing) strains showed no change for blood agar, even after one year on plain agar.

* Moore (1893) described over thirty streptococci mainly from pathologic conditions, emphasizing the time of clearing, etc. Laabs (1910) ranked broth first (above injection) in diagnostic value.

TABLE 6

RECORDS AFTER A ONE-YEAR INTERVAL (TEN-DAY TRANSFERS) FOR VARIOUS STREPTOCOCCI *

Strain	Source	Time Since Isolated	Chain Length (Longest Seen in Sugar Media)	Clearing in Broth in	Agar Colonies	Gelatin, Growth in	Litmus Milk Coagulation in	Color in 10 Days	Blood Agar	Saccha-rose	Lac-tose	Sal-icin	Raffi-nose	Man-nite	Inu-lin
D 16	Dog 1, ecum	1 day 1 year later	30 30	10 da. None	Muggy Muggy	2 days 18 hours	None 3 days	Pink purple Top $\frac{3}{4}$ pink	No color Greenish to hazy	-0.1 0.1	5.5 3.0	3.9 2.5	0.1 0.1	0.7 0.1	0.1 0.0
29	Human throat	1 day 1 year later	100 80	2 da. 2 da.	Curious irreg. center Curious irreg. center	None 2 to 5 days	None None	No change No change	No color Pale green	3.3 4.5	-0.2 0.1	4.5 4.1	0.4 1.7	-0.1 0.1	0.0 -0.1
11	Milk	1 day 1 year later	20 20	10 da. 2 da.	Usual Usual	24 hours 18 hours	24 hours None	Top half purple No change	Greenish to hazy Greenish to hazy	0.0 0.0	0.2 0.3	0.1 2.8	0.0 -0.8	0.2 0.0	0.0 0.1
42	Mastoid abscess	2 years 1 year later	40 200	3 da. 5 da.	Thin, irregular margin Usual to thin margin	None 5 days	2 days 2 days	Pink; H ₂ O side Pink	Greenish to hemolysis Pale green to hazy	5.1 2.8	4.5 2.8	0.2 0.1	4.6 2.5	0.1 -0.1	0.4 0.3
59	Rheumatic lesions	1 year? 1 year later	30 6	3 da. 10 da.	Usual Usual	2 days 18 hours	3 days 3 days	Pink; H ₂ O side Pink	Greenish Pale green to hazy	5.7 3.6	4.8 2.9	5.1 3.2	0.2 0.3	3.7 2.3	0.3 0.2
69	Horse 2, feces	2 days 1 y ^r -ar	20 6	2 da. 2 da.	Muggy to thin margin Flocculent to usual	2 days 2 days	None None	No change No change	Greenish No color	5.6 3.9	0.3 0.1	0.0 3.5	0.1 0.0	-0.1 0.0	-0.1 -0.2
82	Horse 5, feces	1 day 1 year	12 12	5 da. None	Usual Muggy	2 days 18 hours	None None	No change No change	No color to hazy Hemolysis	3.3 2.4	3.7 3.0	5.2 4.1	0.4 0.4	4.2 3.6	0.1 0.1
88	Human feces	1 day 1 year	8 8	None None	Muggy ? Muggy	None 18 hours	2 days 3 days	Top $\frac{1}{2}$ pink Pink; H ₂ O side	Hemolysis Hemolysis	0.4 0.2	4.5 3.1	5.3 3.8	0.5 0.1	2.9 2.3	0.1 0.0
251	Dog 5, small intestine	1 day 1 year	80 100	2 da. 2 da.	Usual Usual	None 15 days	2 days 2 days	Pink Pink	Hemolysis Hemolysis	3.0 3.5	2.6 3.3	2.6 3.4	0.2 0.2	0.2 0.2	0.1 0.2
245	Dog 5, stomach	1 day 1 year	100 60	5 da. 2 da.	Usual Usual to thicker edge	10 days 10 days	2 days 2 days	Pink Pink	Hemolysis Hemolysis	3.1 2.9	2.6 3.5	2.4 3.1	0.3 0.3	0.0 0.2	0.0 0.3

* But ten representative strains are given here; the generalizations, etc., are based on the whole thirty-one strains.

CONCLUSIONS REGARDING CONSTANCY IN RELATION TO AGE

Periods of cultivation on artificial media may produce or be characterized by changes in physiologic activities.

The quantitatively measured fermenting powers—63 to 77 percent constant for periods of one year or less—are fully as constant as the qualitatively measured results obtained with gelatin, broth, litmus milk, and on plain and blood agar plates.

When fermentative changes occur, they seem sufficiently related to the former fermentative complexes to indicate three main lines of development of fermentative powers: (1) a lactose-mannite line, (2) a saccharose-raffinose line, and (3) a smaller and more variable salicin line.

The fluctuation within these three groups serves to emphasize the integrity of these groups; this, with the rarity of cross-connections between the three groups, emphasizes the probable ancestral limitations of these groups or series.

CONSTANCY AS AFFECTED BY TEMPERATURE AND MEDIA

The variations shown by streptococci during a long period of cultivation do not necessarily affect the diagnostic value of the characters they may exhibit when freshly isolated. Even if we think of the streptococci as definitely or permanently modified by the environments from which they have been isolated, that increases (and does not lessen) the possible diagnostic value of such characters.

The point of real importance is, rather, are there any differences of laboratory procedure (during isolation, pending the final tests, etc.) that may modify the final results, and so explain the differences in the results obtained by various workers. And these results are very different; for example, 8 percent of the forty-six milk strains described by Hilliard (1913) ferment salicin, but 77 percent of my 133 milk strains are salicin fermenters.

If some of the conditions attending identical or differing results in any one laboratory could be found, we might know where to look for the factors that must be considered in comparing the results obtained in different laboratories. Upon investigation, definite differences in technic came to light. Omitting age (already discussed), we may treat these under two* main topics, temperature and media.

* Variation in the amounts used for inoculating the Gordon test substances has also been mentioned as possibly causing some of the differences in these Gordon results. To test this, forty tubes were inoculated with varying amounts: three loops, one loop, and the tip of a fine stab needle. The titration results three days later showed no differences worth noting. Sometimes the result with the smaller amount was 0.1, 0.2, or even 0.3, ahead of the record for the larger amount.

TEMPERATURE

(a) Cultures in some laboratories were stored (after incubation at 37 C.) in the ice box; in others, they were kept at room temperature. To see the effects of such different temperatures on the later (37 C.) fermentations, duplicate cultures of ten strains were kept from four to seven days at the two temperatures 20-24 C. and 6-10 C. and inoculated directly into the Gordon media. In the titrations at the end of the usual three-day period, no differences were found.

(b) In most laboratories, practically all the strains described were grown on agar at 37 C. Gelatin was occasionally used, however. Here we have a double difference of media and temperature, but ten duplicate cultures used experimentally to find possible modifications due

TABLE 7
EFFECT OF TEMPERATURE ON THE FERMENTATIVE REACTIONS OF STREPTOCOCCI

Strain	Source	Tempera- tures C.	Saccha- rose	Lac- tose	Sal- icin	Raffi- nose	Man- nite	Inu- lin
38	Blood.....	Original 37	5.2	4.8	5.3	0.7	3.4	0.4
		40.....	2.7	2.7	2.9	0.2	2.4	0.1
		Retest, 37..	3.2	6.0	6.8	0.3	5.2	-0.1
42	Abscess.....	Original....	5.1	4.5	0.2	4.6	0.1	0.4
		40.....	1.7	1.7	0.0	1.9	0.1	0.1
		Retest.....	4.1	5.6	0.2	6.2	0.1	0.3
29	Human throat.....	Original....	6.9	0.4	5.7	0.2	0.2	0.1
		40.....	4.5	0.0	3.5	0.0	0.0	0.1
		Retest.....	4.3	-0.1	4.3	0.1	0.1	0.0
7	Dog's throat.....	Original....	4.7	1.4	6.0	0.5	4.0	0.2
		40.....	3.5	1.6	3.6	0.0	2.7	0.0
		Retest.....	3.7	3.6	8.6	0.2	6.0	0.3
4	Dog's stomach, ear- diac	Original.....	0.0	5.4	4.7	0.1	0.5	-0.1
		40.....	0.1	3.7	2.8	0.0	-0.1	0.2
		Retest.....	0.5	6.7	5.0	0.3	0.9	0.2

to such differences in preliminary treatment gave practically identical results in the Gordon media (and also in blood, litmus milk, etc.). Hilliard found that milk streptococci gave the same fermentative result when grown at 20 C. and at 37 C., while throat streptococci at 20 C. did not readily use any of the sugars utilized at 37 C.

(c) Differences slighter than this may affect the results appreciably. There is some indication in my work that 35 C. to 37 C. is a more favorable range than 37 C. to 38 C.

Temperatures above 37 C. are apparently less favorable. One very hot night the temperature in our incubator room exceeded 37 C.

Sometime between 11 p. m. and 5:30 a. m., the temperature reached 40 C. This was about the middle of the three-day incubation period for the Gordon tubes. When titrated later, they all yielded records decidedly lower than those of earlier titrations of the original strains, and also below those obtained in later work with subcultures of the original strains. Table 7 shows for a few of these strains the marked depression due to this period of unfavorable temperature. The effects were not only marked but consistent throughout the entire set of 40 strains in all six media.

Other strains (12 in all) incubated on agar at 40 C., and then inoculated into Gordon media, did not differ materially from their original records. With regard to temperature, therefore, it would seem as if the temperatures during the period of growth, observation, or measurement were most important; differences in temperature preceding these final tests are apparently not important, so long as they are not so extreme as to kill the organisms. (It is probable, of course, that the sugar and related Gordon media act as revivifying influences.)

MEDIA

Most of the differences in procedure, however, are usually questions of media—of concentration, acidity or alkalinity, or additional nutrient substances, etc. For example, some workers used plain agar throughout for isolation; a few invigorated all cultures preparatory to the Gordon tests by dextrose agar, North's media, etc.; others used selective media, such as lactose litmus agar, for isolation of their strains; most of the workers used meat extract instead of meat for all the preliminary media as well as the final test or Gordon media.

Special media used for isolation of streptococci seem to act selectively with regard to the streptococci; for example, lactose litmus agar yields a series of strains with a non-typical proportion of lactose fermenters. The main problem here, however, is the effect of media upon the subsequent Gordon reactions. For these experiments duplicate cultures of ten to thirty strains (listed as follows) were cultivated, and then inoculated into the usual Gordon media:

(a) On agar (all agar, broth, and gelatin throughout made from meat, unless meat extract is definitely mentioned) and on gelatin (1 to 3 days).

(b) On agar and in (autoclaved) milk (1 to 10 transfers, 3 days apart).

(c) In (Arnold) milk and in meat extract broth (1 to 3 transfers).

- (d) On plain agar and on dextrose agar (1 to 8 days).
- (e) On plain agar and in dextrose broth (1 to 10 transfers, 1 day apart).
- (f) In plain broth, in sugar broth, and on plain agar (10 transfers, 1 to 3 days apart).
- (g) In serum broth and in dextrose broth (3 transfers).
- (h) In plain broth of varying acidity 0.5, 1.5, or calcium carbonate (10 transfers, 3 days apart).
- (i) On plain agar, in sugar-free broth, and in each of the six Gordon media (10 transfers, 3 days apart).
- (j) In meat extract broth, and in meat broth, and also in Gordon media made of meat extract and of meat.

None of these first nine experiments (a-i) yielded any differences worth noting in the final Gordon titrations which followed. Experiments (b) and (c) were included to see whether they accounted for the high acidities obtained in my early work with milk streptococci. The differences here, also, are too slight to be of importance. Milk, as a preliminary culture medium, was therefore not entirely, if at all, responsible for the extremely high acid results reported earlier (1912) for my milk streptococci.

The 174 strains studied under (h) indicate (1) that $+0.5$ is a more favorable initial acidity than $+1.5$; and (2) that calcium broth is not more favorable for growth than broth without the addition of calcium. Nearly 200 tubes incubated for ten days and streaked for viability showed a lower percentage (76 percent) of viable strains than did plain broth (93 percent). This is not in accord with the common practice of bacteriologists, especially those working with strains of pathologic origin; the alkaline reaction of the body tissues and fluids may explain this accepted practice. The condition in part of the alimentary canal, at least, is decidedly higher in acidity; Escherich reports also a difference in the acidity of infant and adult stools. All this will explain, probably, why calcium broth presented apparently no advantages for my strains, many of which came from the alimentary canal and strictly saprophytic, or not strictly parasitic, sources.

My results for (i) do not indicate any real or definite influence* due to the lack of sugars or to the presence of saccharose, lactose, and

* In many cases certain of the Gordon media (not utilizable) showed less indication of growth (gross and microscopic) than sugar-free media. This is explained by Hilliard in a letter, 1914, in which he says that "the added organic substance has acted as a bacteriostatic agent." The larger number of swollen organisms in such cases (when compared with sugar-free smears) would indicate that the effect of the Gordon substance may be something more than bacteriostatic.

such related substances. After one month on sugar-free media or on the Gordon media, the tenth transfer of each strain was again inoculated into its respective substance (salicin into salicin, inulin into inulin, etc.). These special media were incubated three days as usual and titrated in the customary manner. A table is given for part of these strains (Table 8), as many workers have obtained effects interpreted as directly due to such preliminary treatment.

TABLE 8
EFFECT OF PRELIMINARY TREATMENT WITH REGARD TO SUGARS

Strain	Source	Preliminary Media	Preliminary Period	Saccharose	Lactose	Salicin	Raffinose	Mannite	Inulin
7	Milk.....	Agar.....	3 wk.	3.8	4.7	4.4	2.9	2.9	0.2
		Sugar free....	3 wk.	3.9	4.4	4.9	3.3	2.9	0.3
25	Human feces...	Agar.....	3 wk.	4.4	4.3	4.6	0.4	3.9	0.5
		Sugar free....	3 wk.	4.4	3.4	5.2	0.4	3.4	0.4
26	Human feces...	Agar.....	3 wk.	4.1	3.5	4.7	0.3	3.7	0.2
		Sugar free....	3 wk.	2.2	3.6	5.5	0.4	3.5	0.4
29	Human throat	Agar.....	1 mo.	4.3	-0.1	4.3	0.1	0.1	0.9
		Dextrose agar	1 mo.	4.5	-0.1	4.2	0.1	0.1	0.0
8	Milk.....	Agar.....	5 da.	3.8	1.0	4.4	3.1	0.0
		Dextrose agar	5 da.	3.7	0.9	5.0	0.0	3.1	0.2
20	Cat, abscess...	Agar.....	5 da.	1.4	3.8	4.1	1.4	2.9	1.6
		Dextrose agar	5 da.	1.3	3.5	4.5	1.1	2.9	1.5
21	Cat, abscess...	Agar.....	5 da.	1.3	3.5	4.2	1.5	2.9	1.6
		Dextrose agar	5 da.	1.2	3.5	4.5	1.3	2.8	1.6
21	Cat, abscess...	Agar.....	1 mo.	0.1	0.3	0.1	0.1	0.2	0.2
		Dextrose agar	1 mo.	0.0	0.3	0.1	0.0	0.1	0.2
3	Blood.....	Agar.....	1 mo.	3.5	4.1	4.4	2.5	2.2	3.0
		Respective Gordon media	1 mo.	3.3	2.8	4.4	2.6	2.9	2.8
18	Milk.....	Agar.....	1 mo.	4.1	3.4	4.6	3.2	3.2	0.2
		Respective Gordon media	1 mo.	2.3	3.8	4.6	3.1	3.0	-0.1
30	Chicken, blood	Agar.....	1 mo.	3.8	2.9	3.4	0.2	1.3	0.4
		Respective Gordon media	1 mo.	4.0	1.9	1.9	0.8	1.4	0.7
24	Human feces...	Agar.....	1 mo.	3.8	4.0	5.2	0.3	3.4	0.4
		Respective Gordon media	1 mo.	4.1	4.4	5.6	0.3	3.3	0.3

My strains showed for the substances fermented no gain in the amount of acid formed; and no strain learned to ferment any of the substances which it had not previously fermented. And the few changes apparently induced in later similar experiments often appeared simultaneously or later in agar controls.

There is nothing in the work of Klotz (1905-07), Neisser (1906), Penfold (1910), and Walker (1910) to indicate that this is not really a question of vigor of growth rather than of the inducing of specific fermentative powers. Penfold (1912) used single-cell cultures, so that such variations or mutations cannot be dismissed summarily as due to "mixed strains."

Then, too, the differences attributed to sugar-feeding are not always consistent; for example, their strains do not progressively gain the power to ferment a given substance, as one might expect if such gain were due to continued subjection to that substance; and strains using a given substance may apparently suddenly cease using that substance. These discrepancies or irregularities, I think, are at least partly to be explained as follows:

(1) Sugar media often kill off streptococci within a short period.* Thirty different strains yielding (after three days) heavy growth in the Gordon media and characterized by high acidity (3.6 to 5.3), were streaked (two to three loops each) on agar; about 61 percent failed to grow on the agar plates. The accumulated acids were probably mainly responsible for these germicidal results shown in saccharose, salicin, and similar media, tho growth was obtained from a few of the tubes with very high acidity (4.7, 5.3). (2) The explanation of discrepancies and irregularities when this work was carried on in meat extract media, is probably found under the next experiment, (j).

(j) The comparative effects of meat and meat extract have been described in an earlier (1913) paper. Some strains grew very well, at first, in meat extract broth; but after two or three successive transfers in meat extract broth, one or more strains in each lot (of six to twelve strains) died or had to be revived by more favorable food material. If, however, the strains remained alive, they did as well as their controls when they were inoculated into the usual Gordon media (made from meat). More striking was the difference between Gordon media made of meat extract, and Gordon media made in the usual way, from sugar-free meat broth. Here, even tho the two lots of Gordon media were inoculated from the same agar slants, a great variation was found in the titration records.

Strains that gave a high acidity in Gordon media made from meat often fell to 1.0 or even to 0.8 and 0.7 in meat extract † Gordon media. This range includes the neutral point for litmus, and it readily can be seen that strains classed as fermenters when meat was used would often give negative results with litmus in meat extract media.

Almost all workers on these fermentative activities of streptococci have used meat extract media for these tests. This surely

* In this connection it may be of interest to add that even plain broth and milk cultures (ten-day incubations) differ in this respect. Of the 85 strains, 88 percent survived the ten-day period in plain broth, and 54 percent in litmus milk. Milk usually had the higher final acidity, 2.3 to 3.0; that of the plain broth ranged from 1.2 to 1.8. Most of the Gordon media failing to give indications of live organisms ranged from 4.5 to 5.6.

† This indicates a great difference in physiologic activities, and may therefore have an important bearing upon the substitution of meat extract for meat (infusion) media in the production of toxins, vaccines, and similar preparations.

accounts for many of the irregularities one finds when trying to compare the records of various investigators, altho several state that their results were the same, whether measured with litmus (or Anrade's acid fuchsin) or by titration with phenolphthalein. This may be true for any individual worker using only meat extract media or meat media throughout. But I believe it is impossible to compare satisfactorily, if at all, strains grown in the two kinds of media, especially where there is also a difference in the indicators (e. g., litmus and phenolphthalein).

Related to this probably are the curious results obtained by Floyd and Wolbach. Of 247 strains, mainly from pathologic conditions, over 60 percent failed to ferment any of my six Gordon media. They used neutral red as an indicator, and while their control tests indicated that this was extremely sensitive (to 0.25), it is hardly possible that there is not some difference in technic to explain why their strains (one from a sample, evidently) differ so markedly from those of other investigators.

Another cause of discordant results in the work of many investigators is probably the variety of media used for the Gordon tests. Hiss serum water, meat infusion broth, meat extract broth, and peptone solution are the principal foundation substances to which the Gordon test substances are added. One worker uses meat infusion broth without making it sugar-free, subtracting the results of control tubes of the meat infusion broth. This seems hardly reliable, as the acid formed from the muscle sugar may influence the cleavage of the Gordon test substances. Rogers and Dahlberg add dibasic phosphates to their media, feeling that it favors materially the acid production. Their records compare more favorably with my meat media records than those of any other workers.

Still more important in producing the lack of uniformity is the combined effect of such depressing influences as accumulating acids, and such unfavorable media as meat extract, just described under experiments (i) and (j) in the final Gordon tests.

Few of my strains (on meat-infusion agar) showed any tendency to die off. Altho several workers state specifically that their strains were alive when inoculated into the Gordon test media, the conditions under which many of them carried their stock cultures were, in my experience, warranted to subject them to such depressing influences.

While I gained no differences due to such preliminary treatment, it is doubtless true that prolonged subjection to one or both of these unfavorable conditions would (1) yield less vigorous strains that might not recover equally in the various Gordon media, or (2) would act selectively on the strains under cultivation. (Bergey (1912), for example, says that one or more of each set of his strains died at each subtransfer.)

CONCLUSIONS REGARDING CONSTANCY AS AFFECTED BY TEMPERATURE
AND MEDIA

Summarizing the experiments listed, we may draw two conclusions: Tho the life of any strain is apparently shorter, and more uncertain, in meat extract media, and in media of high acidities, such "previous conditions of servitude" do not appreciably affect the later fermentative activities of streptococci. The fermentative activities are markedly depressed (1) by increased temperature during the Gordon media tests, and (2) by the use of meat extract instead of meat for making the Gordon media.

MORPHOLOGIC VARIATIONS

Tho constancy is here discussed from a physiologic standpoint, there are certain induced morphologic variations that may be of interest, tho they seem to be temporary only. No matter how extreme they may be, they disappear entirely with the conditions that evoked them.

The most marked morphologic modifications observed in this study are produced by dextrose and the six substances (saccharose, etc.) used for the Gordon media. Of the 767 strains studied, smears were made of the Gordon media just before titration for about 600 strains. These, with the duplicates and repetitions that were made, make a total of over 8,000 Gordon slides. While the original purpose was to confirm the purity of the cultures, it soon became noticeable that there were certain differences in the morphologic characteristics that were as indicative of a given medium as of the various streptococci themselves.

Most strains which grow well in these Gordon test substances tend to show the following changes:

(1) Marked increase in chain length or a very marked decrease. A strain ranging normally in plain broth from 30 to 20 units to a chain usually shows occasional to many chains of 60 to 100 (or more) units, with varying numbers of shorter chains of 30, 20, 50 units, etc.; or a chain ranging from 8 to 12 units will often, in Gordon media, have 8 or 10 as its highest limit, with almost all of the organisms in combinations of 2 and 4 units, or even reduced to 2's and single units.

(2) With heavy growth, there is also a tendency to form new organisms at right angles to the usual plane of division, so that the ordinary linear or chained arrangement is often broken by one or more cells which have divided in this way. Escherich (1886) pictured

intestinal streptococci characterized by such division. The short-chained forms from the intestines and feces (and they are usually short-chained) are very readily induced to show this less common type of division. When this change is accompanied by a tendency to reduce the chain length, we have an accumulation of small clumps of three to six or eight organisms that present anything but the customary streptococcus slide.

(3) The morphology of the individual cell may be likewise affected. In media not utilized, some of the chains are usually of full or increased length, and there is usually a small proportion of swollen organisms (rounded and elliptical) either in short chains or interposed here and there in chains composed mainly of normal organisms. When the media are utilized, there is, besides this swelling, a distinct tendency to abnormal shapes. The cells are often elongated, two to four times the natural length. This is probably due to a failure to divide promptly; careful focusing will usually bring out some indication of the units represented in the rod-like objects. Often, however, more varied forms are seen—organisms which may be actually pear-shaped, club-shaped, or obtusely diamond-shaped. These changes are most marked in mannite, tho they may occur in the other media. The swollen and undivided forms, without these abnormal shapes, are more often found in saccharose, lactose, or salicin; perhaps less often in raffinose. A normal appearance is effected at once, however, by transplanting a mannite culture to plain broth; the abnormal characters reappear as promptly when mannite feeding is resumed.

Some of the intestinal forms show on isolation a tendency on the part of the units in the occasional pairs to lie so that one side of the pair forms an angle of less than 180 degrees. This tendency is increased by Gordon media which the strain can utilize.

Changes in morphology have of course been previously reported for other bacteria, due to hypertonic salt solutions, phenol, etc. I have not seen any references to such changes in streptococci with sugar media, tho it must be well known.

In several instances, dextrose cultures showed decided capsules (Hiss method) and the plain broth cultures did not. This was marked in some of the strains possessing wide, hazy capsules. Strains having apparently no capsules were grown in dextrose broth; two-day dextrose broth cultures of these (24) strains showed very decided capsules. Following this lead, I made forty duplicate inoculations (1)

into serum broth and dextrose broth, and forty others (2) into plain and into dextrose broths. The results were not consistently in favor of the serum broth; dextrose broth seemed more favorable than plain, tho not without exception. Altho dextrose and serum broth may bring back a lapsed capsule, the indications are that they do not cause a capsule, except as they encourage a more vigorous growth.

Finally, the common statement that reduction in chain length of streptococci follows cultivation on artificial media might be referred to here. I have noted no tendency of the kind in nearly 150 strains continued on artificial media for periods ranging from fifteen days to nearly two years. Practically every strain (including those from pathologic conditions) gives in plain broth or in the Gordon media the chain length originally given in the respective media. If meat extract had been used for my work, I feel sure this would not have been true, and I might then agree with the popular statements regarding not only the reduction in chain length, but the difficulty in keeping the streptococci alive.*

CONCLUSION

Morphologic characters often vary greatly with the media, but such induced variations are apparently temporary only.

CONSTANCY AS AFFECTED BY VARIATIONS IN ENVIRONMENT DESIGNED TO INDUCE CHANGES IN STREPTOCOCCI

The wider fermenting range of the saprophytic, intestinal, and fecal streptococci when compared with those known to be of pathogenic origin, and the intermediate character of many of the mouth streptococci, suggested the following questions: What is the natural or usual habitat of the non-fermenters, the low fermenters, and of the strains fermenting but one or two substances? Do such strains fail to get past the throat region? Or do such strains gain fermentative power by a stay in the alimentary canal, as we have already seen that they may when kept on artificial media?

In this connection, several experiments were made with streptococci peculiar or limited in their original fermenting powers to see whether these powers could be materially and permanently changed or increased by subjection to various influences found in the animal

* I had practically no trouble in this connection. Some from blood, etc., were exceedingly difficult to isolate, growing well in broth, but absolutely refusing to grow on agar (or vice versa). But once isolated, I have lost of the hundreds studied less than ten strains of those I desired to continue.

body. These experiments fell into two lots: (1) those in vitro subjecting streptococci to such substances as saliva, milk, and intestinal extract; and (2) experiments with living animals in which streptococci were (a) fed by the mouth (with streptococci-free food or in celloidin capsules) or (b) inserted in capsules directly into the intestines. A complete list of these experimental environments follows:

1. Hydrochloric acid.
2. The alimentary canal, streptococci having been given (a) with food or (b) in celloidin capsules, the capsules being inserted directly into the small intestine and body cavity (regained at autopsy), or given by mouth (regained from the feces).
3. Milk, raw, and heated to various degrees.
4. Intestinal extract (a) from young kittens and (b) from fetal pigs.
5. Pure cultures of other bacteria.
6. Saliva, (a) normal and (b) heated.

HYDROCHLORIC ACID

Hydrochloric acid is listed here because it is one of the conditions suggested as probably determining the type of bacteria found in the intestines, either by acting as a selective agent or by modifying the streptococci during their stay in the stomach.

Ten strains were subjected (1) to water plus 0.2, 0.4, and 0.6 percent hydrochloric acid, and (2) to broth, originally neutral to phenolphthalein, to which the same amounts of acid were added. The broth plus acid strains lived longer, often surviving fifty minutes in 0.6 percent, seventy minutes in 0.4 percent, and 120 minutes in 0.2 percent. The broth plus acid medium represents probably more nearly the condition in the stomach than does the water plus acid. Strains regained from these broth-acid conditions were not impaired in their power to ferment dextrose.

Since streptococci survived 0.2 to 0.4 percent HCl for from ten to seventy minutes, and since water alone is immediately passed through the pylorus, and carbohydrates sometimes begin to leave the stomach ten minutes after such food is taken, these results show that the effect of hydrochloric acid on the streptococcic flora of the intestine must be much less than is usually implied. It seems wise, therefore, not to accept too readily current statements concerning the germicidal action of the gastric juice on ingested bacteria.

A STAY IN THE ALIMENTARY CANAL

Streptococci were also fed to several dogs kept on streptococci-free food (milk, bread, oatmeal and water). A litter of nine hound puppies about two months old were studied for a period of nearly five months.

First, an effort was made to find the range of throat and fecal streptococci characteristic of these dogs. In this work, seventy strains of streptococci were isolated and studied in litmus milk, gelatin, blood agar, Gordon media, etc. The dogs were then kept for nearly three months on streptococci-free food. No attempt was made to free the air of streptococci, tho various precautions were taken to control the introduction of new streptococci (see appendix for other details).

After six weeks on streptococci-free food, one puppy was examined after death to determine the characteristic, persisting streptococci. The remaining eight puppies were divided into three groups. Three puppies kept in one run were fed Strain 29, two in another run were fed Strain 11, and the other three were kept in a third as controls. Strain 11 was a non-fermenting strain of not very pronounced character; it was chosen to see whether it might not persist as a non-fermenter in the stomach, intestines, etc. Strain 29 was an unusual one, chosen because it was not at all like the alimentary streptococci of the animals previously examined (six cats, three dogs, one pigeon, two chickens), nor like any fecal streptococci found in the 160 odd strains previously isolated from man, horse, cow, cat, and dog feces. It differed from all stomach and intestinal streptococci in that it formed rather long chains (thirteen to forty units on plain broth, 100 to 200 in sugar media), had an unusually broad, hazy capsule (Hiss stain), cleared rapidly in plain broth (with numerous fine, soft granules), did not affect litmus milk, and (in my experience) had an unusual fermenting combination, saccharose and salicin only. A throat strain often shows one or more of these characters, but not the whole complex; none of my earlier studies of the alimentary and fecal bacteria of cats and dogs (about 200) showed any streptococci at all resembling this strain.

The streptococci were poured on sterile (170 C.) crusts, which were fed to the respective dogs. Eight days after the third and last feeding (seven-day intervals), the dog was examined after death and samples (thirty to forty-five) taken from various parts of the alimentary canal (mouth to large intestine). From these, isolations were made, and from twenty to twenty-five strains selected for study in the various media—milk, blood agar, gelatin, Gordon media, etc.

It was found impossible to keep the outdoor cages entirely free of flies, and so but six of the dogs were examined at autopsy (two for Strain 11, two for Strain 29, and two controls). No non-fermenting strains were recovered from the dogs fed with Strain 11. The two fed with Strain 29 were each killed as usual eight days after the last dose of streptococci.

Strains which I feel sure were the original 29 were gained from one or more samples from the esophagus, cardiac sphincter, fundus, and duodenum. Strain 29 had apparently retained its medium chain

length (often 20 to 30 units), its broad capsule, and its way of clearing in broth—all of which are unusual for alimentary streptococci, at least below the cardiac end of the stomach. It had changed however in several striking ways, having now gained the power of coagulating milk, of fermenting lactose, and of hemolyzing blood—the latter not being considered common in intestinal strains.

I realize perfectly that many would feel that it was very unlikely that I had ever regained my original strains. I have no doubt of it myself, however, having experimented with Strain 29 for over four months in an endeavor to induce certain changes and knowing how much unlike the usual alimentary streptococci its retained characters are. Most striking of all was its appearance on agar, a curious, thickened to hardened or branching center, with a thin margin, and, with age, a concentric-ringed appearance, the margin finally becoming (under certain conditions) thicker. This is not at all characteristic of intestinal streptococci.

The next summer similar feeding experiments were made with two ten-day kittens. They were kept under much more carefully controlled conditions, but they need not take space here, as, after autopsy, the control cat yielded the usual range of streptococci, while the streptococci-fed cat (also Strain 29) did not yield streptococci in any of the twenty samples taken, even tho the cat was fed a 1 c.c. dose twenty-four hours before it was killed. This absence of streptococci is most unusual, if judged from the eight other cats previously examined.

In the later work, known streptococci were grown in little celloidin capsules (or parchment-tipped, glass capsules) and inserted in the intestines of two healthy dogs. Duplicates were at the same time placed in the peritoneal cavity. Dr. Max Pickens generously performed the operations. One dog died in two days. The other was killed eight days later. In both cases, the capsules had been invaded by foreign organisms and showed no streptococci.

Later, a better method of preparing the capsules was found, and the next year a collie dog was used in another feeding experiment. This dog was fed three meat-wrapped celloidin capsules, each containing dextrose cultures of the same Strain 29. After three days, the capsules were recovered from the feces, opened, examined microscopically, and streaked on agar plates. The contents of the three capsules when titrated showed four times the acidity of control capsules; exchanges with some of the alimentary canal substances were therefore assured. Two capsules were found to be sterile; the third contained a pure culture of streptococci.

The streptococci regained from this capsule had gained* (Table 9) the power to ferment lactose, raffinose, and inulin. They also turned litmus milk pink and coagulated it. These recovered Strains 29 still cleared in broth, and, on dextrose feeding, showed the very broad, hazy capsules. Even more striking was the retention of the peculiar character of the agar colonies. There is no room for doubt as to the verity of the strain in this case. And it must be admitted that the changes claimed for the regained Strain 29 in the original feeding experiment described are at least probable. Seven fishings were made from the celloidin capsule streak plate, and all agreed in the characters described. Two of these fishings were continued on agar in the usual manner for ten-day transfers and when tested (5 months later) in their Gordon activities were constant in all the characters acquired during their stay in the alimentary canal.

TABLE 9
STREPTOCOCCUS 29 BEFORE AND AFTER A STAY (IN CELLOIDIN CAPSULES) IN THE ALIMENTARY CANAL

	Agar Colony	Broth	Litmus Milk	Blood	Saccha- rose	Lac- tose	Sal- icin	Raffi- nose	Man- nite	In- ulin
Strain 29 originally and at Time of Exper- iment	Radiate center; thin margin; concentric ringed with age	Clearing in 2 days	No change	No color	+	0	+	0	0	
Strain 29 Regained from Capsules	Radiate center; thin margin; concentric- ringed char- acter empha- sized	Acid, co- agulated	Hem o- lyzed	+	+	+	+	0	
					4.7	0.1	3.5	0.1	0.0	
					4.6	4.5	4.5	4.3	0.0	

This increase by Strain 29 in fermentative range is in line with the greater range of fermentative activity shown by intestinal forms.

MILK

Meanwhile, Rosenow had described a transformation produced on *Streptococcus mucosus* by milk baths of raw, unheated, sterile milk. Strains after "one soaking" in sterile, unheated milk regained "disappearing characters" (such as the capsule) characteristic of septic sore throat streptococci. These effects were less marked with incubated milk, still less when pasteurized milk was used, and absent with auto-claved milk.

* In this line, it is interesting that five months after this experiment and twenty months after isolation, the stock culture of this Strain 29 also began to ferment raffinose and inulin, tho not lactose (see Table 11).

I secured a few dozen samples of sterile milk, milked directly into sterilized tubes, from the Walker-Gordon Dairy at Plainsboro, N. J. Two sets were kindly given me, one set of 16, in which 14 remained sterile, and a second set of 70, including 57 sterile tubes. With these I tried out several duplicates of Strain 29 to see whether this strain, which in many ways resembles the septic throat streptococci described by several workers, could be induced to change in any way. The only effect was upon the agar plate characters, more colonies showing the concentric, thicker-ringed type instead of the original curiously centered type.

INTESTINAL EXTRACT

To test still further the effect of various conditions or substances found in the alimentary canal, seven strains were subjected to intestinal extract from young kittens, and, later, two other strains to intestinal extract from fetal pigs. No changes were obtained in the first experiment. Tho freshly made, the extract reduced lactose but feebly.

TABLE 10
STREPTOCOCCUS 26 BEFORE AND AFTER SUBJECTION TO INTESTINAL EXTRACT

Condition	Agar Colonies	Gelatin	Litmus Milk	Blood Agar	Saccharose	Lactose	Salicin	Raffinose	Mannite	Inulin
Before Aug. 21	Usual	No growth in 10 days	No change	No color	+ 2.5	0 0.0	0 0.1	+ 2.1	+ 2.0	+ 1.8
After Sept. 1	Usual	In 18 hours	Slightly acid; not coagulated	Slightly greenish	0 0.2	+ 2.0	+ 4.2	0 0.1	+ 3.0	0 0.1

The pig extract, obtained from five fetal pigs sent by Jacob Dole of Buffalo, reduced lactose readily. Several tubes of this extract were made and examined for bacteria. In none were streptococci ever seen; large cocci occurred in a few tubes, and one or two contained rods. Capsules containing twenty-four-hour dextrose growths of the two different streptococci (29 and 26) selected were suspended in this extract. After two days, these capsules were examined and streaked as previously described. Each one yielded a pure culture on the stained slide, and apparently streptococci only on the agar streak plates. Strains were isolated from these streak plates and tested in the usual media—gelatin, blood agar, litmus milk, Gordon media, etc. In one only, Strain 26, were changes found (Table 10). These were so peculiar that an unused tube of fetal extract was examined for sterility,

new capsules of Strain 26 prepared, and the experiment repeated, with the same result.

All of the eleven fishings tested had lost two of their original powers, saccharose and inulin, and gained two new ones, lactose and salicin. The complex thus became lactose-salicin-mannite, a common intestinal complex.

SALIVA

Following this, experiments with saliva were tried in a similar manner. Table 11 gives the results for two of these strains after subjection (in celloidin capsules) to saliva.

TABLE 11
TITRATION RECORDS OF STRAINS OF STREPTOCOCCUS (IN CELLOIDIN CAPSULES) SUBJECTED TO SALIVA

	Saccharose	Lactose	Salicin	Raffinose	Mannite	Inulin
Strain 29 originally	+	0	+			
	4.7	0.1	3.5	0.1	0.0	0.1
	+		+		+	
	3.9	0.0	4.8	0.2	2.5	0.1
Strain 29 subjected	+	0	+		0	+
to saliva (1 da.),	4.0	0.1	4.3	3.4	0.1	4.7
showing various	+	0	+	0	0	+
resulting types	3.4	0.1	4.6	0.0	0.2	4.9
	+	0	+	0	0	+
	2.8	0.1	2.5	0.1	0.1	4.8
	+	0	+	0	0	+
	3.4	0.0	2.4	0.1	0.1	5.0
Changes appearing	+		+	+		+
in Strain 29 after	5.4	-0.1	4.7	2.7	0.2	4.9
20 mos. constancy	+	0	+	+		+
	5.4	0.2	4.6	3.1	0.3	4.8
Strain 29 subjected	0	0	+	+	0	+
to another sample	0.1	0.1	4.5	3.3	0.2	5.1
of saliva, while	+	0	+	+	0	+
still constant to	5.5	0.1	4.5	3.2	0.3	4.2
original fermenta-	+	0	+	+	0	+
tive complex,	5.3	0.3	4.5	3.1	0.0	4.8
yielding	+	0	+	+	0	+
	5.2	-0.2	4.3	3.0	0.2	3.4
Strain 42 originally	+	+	0	+	0	0
	2.2	2.5	0.1	2.6	0.5	0.2
	+	+	+	+		+
	3.8	3.0	2.7	3.3	0.4	1.3
	+	+	+	0	0	0
Changes appearing	3.5	3.0	3.8	0.1	0.1	0.1
in Strain 42 after	+	+	+	0	0	0
similar subjection	3.3	2.6	2.9	0.1	0.1	0.1
to saliva	+	+	+	0	0	0
	3.2	3.0	3.1	0.1	0.1	0.2
	+	+	+	0	0	0
	2.9	2.5	2.7	0.1	0.2	0.1

In Table 11, it will be noted that in both strains often more than one type resulted, a circumstance suggesting that the buccal conditions were sufficient to initiate change—to upset the original character complex, without fixing one set of characters upon the subjected streptococci.

Duplicate capsules of several different strains of streptococci were treated with heated and unheated saliva (same sample), to ascertain whether the results obtained by Rosenow with heated and unheated milks could be paralleled. Some differences were found, but they were mainly negative, such as the loss of lactose in the unheated saliva strain, while the heated saliva strain retained its lactose-fermenting character. In most of the cases, neither the heated nor the unheated saliva strains varied at all from the stock control. This was probably due to the fact that most of these strains were old stock cultures. I had difficulty about this time in isolating or obtaining elsewhere fresh cultures with limited fermentative complexes; with such strains I should expect differences in the heated and unheated experiments.

OTHER BACTERIA

About a dozen other cultures in similar capsules were then subjected to the influences of other known bacteria (pure cultures). The results were sometimes negative, sometimes a mixture of the characters; and they sometimes showed a character or power not characteristic of either original strain! (Strain 42 subjected to 29, Table 12). I failed to induce or to inhibit hemolysis in these experiments.

TABLE 12
TITRATION RECORDS SHOWING EFFECTS OF STREPTOCOCCUS 29 (IN CAPSULES) ON STREPTOCOCCUS 42

	Saccharose	Lactose	Salicin	Raffinose	Mannite	Inulin
Strain 42.....	+	+	0	+	0	0
	3.1	2.7	0.1	2.7	0.5	0.1
Strain 29.....	+	0	+			
	4.7	0.1	3.5	0.1	0.0	0.1
	+	+	0	+	0	0
	3.1	2.0	0.1	2.7	0.5	0.4
Strain 42 after sub-	+	+	+	0	0	0
jection to 29	3.6	3.3	3.1	0.1	0.1	0.0
(various fishings)	+	+	0	+	0	0
	2.7	3.1	0.2	2.9	0.7	0.3
	+	+	+	+	+	0
	3.4	3.2	3.5	0.1	3.6	0.3

SUMMARY

The experimental work described was undertaken to test the constancy of the streptococci, or to induce changes in their reactions in various media. The experiments fall, as indicated, into two main divisions:

The first division includes those dealing with relatively simple physical or chemical factors or influences, such as temperature,

acidity, and various artificial media. When these did affect the organisms, the effects were usually of a depressing or inhibitory type; these changes, which were usually differences in amount rather than in kind, were apparently temporary only.

The second division includes the effects of such influences as raw milk, saliva, intestinal extract, and other bacteria; these might be described, by way of comparison with those of the first division, as vital, not merely physical or chemical.

The changes produced by these environmental factors differ, not only in being constant, but in being active, rather than merely passive, and in initiating previously unexhibited powers, instead of merely depressing or inhibiting former activities. They may arise as "mutations," when the conditions have been apparently (but probably only apparently) unchanged, as in the changes appearing with age. Or they may appear as responses to varying environmental conditions, to various "vital" stimuli, such as saliva, intestinal extract, substances in the alimentary canal, etc.

The very short time necessary in Rosenow's work with milk gives increased interest to this view of environmental factors as "vital." In his work the amount of change in the streptococci was inversely proportional to the amount of heat to which the milk was subjected.

In connection with these milk changes, it may be of interest that Houston of London (as he told me in 1910) uses very low temperatures for sterilizing his milk media, because he found that high temperatures gave, in a given set of cultures, a lower percentage showing coagulation. Granting that both of these workers were really working with sterile milk, we may point out that in both cases it is what might be called the "liveness" or degree of "liveness" of the milk that seems to be directly associated with the increased activity of the organisms, or the availability of the media.

In this connection, it is interesting that the positive, active changes produced in the foregoing experiments are all due to similar "live" substances—saliva, intestinal secretions, and other bacteria. Unfortunately, the ultimate causes of these changes, whether they imply the inhibition or the acquisition of specific fermenting powers, are in that most speculative field of enzyme action, and beyond the scope of this paper.

It has been shown by various workers (1) that pathogenic strains are more limited in their range of physiologic activities than related

non-pathogenic strains, and (2) that strains may become so limited* by animal injection (e. g., Klotz (1905) for *B. coli*).

My work, on the contrary, emphasizes the broadening of the range of physiologic activities (1) during periods of cultivation under ordinary saprophytic conditions, and (2) during subjection to the influences to be found in the alimentary canal. Since hydrochloric acid (in the percent characteristic of animal stomachs) is ineffective as a germicidal agent, and since strains of limited physiologic ability may persist when bathed in the animal secretions (saliva, intestinal extract) and take on the wider characters of saprophytic streptococci, it is at least probable that such modified, or transformed, pathogenic streptococci may be not uncommon inhabitants of the alimentary canal,† the alimentary canal forming what might be loosely called an "alternate" habitat. This cyclic theory for streptococci of such varying fermentative powers is supported by the occurrence of hemolyzing strains in normal intestines and feces.

CONCLUSIONS

Two types of induced changes are found. The first type embraces quantitative, temporary changes, in which unfavorable temperatures or the presence or absence of suitable food materials affects directly the physiologic activities of the organisms. With sufficient depression of a physiologic power or powers, a quantitative change may appear as a qualitative one, especially when qualitative measurements are applied, as in the litmus test for acids formed. These changes disappear with the condition that evoked them.

The second type of change is not so mechanical, nor so passive, as the first. It is, on the contrary, active, initiatory, and not to be prophesied. It is illustrated, in my experiments, by the marked changes often produced by saliva, intestinal secretions, and other substances found in the animal body. These changes are more varied, qualitative (and often quantitative, also), and apparently rather constant. (All tested (1 to 2 months) were constant in their acquired characters.)

* Holman (p. 293) thinks that the natural invasive streptococci are not sufficiently considered in some of these animal passage experiments. His "regained" streptococci usually fermented salicin only.

† The streptococci of the alimentary canal are usually much shorter-chained than the accepted form for *Streptococcus pyogenes*. Yet two normal dogs yielded throughout the alimentary canal strains that were very like *S. pyogenes* in morphologic characters. These strains had, however, a wide fermenting range, fermenting all six of the Gordon substances, and they did not hemolyze blood.

II. STREPTOCOCCAL CHARACTERS AS RELATED TO ORIGIN OR HABITATS

The work described in the first part of this paper on constancy of the fermentative reactions has two bearings on the possible differentiation of streptococci according to their origins. (1) Such differences, if they exist, might be expected to show after isolation and in the various test media.

(2) Differences induced (e. g., by means of saliva, intestinal secretions) do not necessarily affect the diagnostic value of the Gordon tests and other differential media. If a stay in the alimentary canal, for instance, can induce changes in the Gordon records of streptococci, the possible diagnostic value of such tests is not impaired, but

TABLE 13

HUMAN FECAL TYPES OF STREPTOCOCCI ISOLATED FROM THE SAME INDIVIDUAL AT VARYING INTERVALS THRU ONE YEAR

Human Feces	Type	Saccharose	Lactose	Salicin	Raffinose	Mannite	Inulin
Sample 1.....	A	0	+	+	0	0	0
Sample 2, yielding A and	B C	+	0 +	+	0 0	0 +	0 0
Sample 3, yielding B and	D E	+	+	+	0 0	0 +	0 0
Sample 4, yielding D and	F	0	+	0	0	0	0
Sample 5.....	G	+	+	+	+	+	+
Sample 6, yielding E only							

increased. That is, of course, unless all saliva, all intestinal secretions, etc., act in exactly the same way, yielding (1) the same types therefore in any given animal at all times and (2) the same types from all animals. The experiments already described, with saliva, intestinal extract, etc., contradict such an assumption. Altho there may be a great similarity in the strains isolated from the various regions in a given animal, Table 13 of strains from one individual through a period of several months also contradicts this assumption. Similar variations were found in seven other animals (throat and feces). The one given covers the longest period (12 mo.), and includes seven different fermentative types.

We also know that in disease, and with age, various foods, etc., the flora of the alimentary canal changes very rapidly and completely.

If, therefore, as seems undoubted, saliva and other body secretions have the power evidenced in this experimental work, the ease

or rapidity with which they affect the streptococci, will influence the phase at which we catch throat, stomach, intestinal, and fecal streptococci. But having isolated them in a definite way (e. g., with extract or with meat media, or with or without selective media, such as lactose litmus agar) we may expect the results in various laboratories to be comparable.

With the range in a given individual as varied as in the case described in Table 13, it looks as tho the normal ranges of the streptococci (mouth, fecal, etc.), from different species (cat, dog, horse, cow, and man) must necessarily overlap.

Still, hoping to verify the predictions of earlier workers, I have studied 767 strains of streptococci isolated from various sources. Usually one to three were taken from each sample; in animal autopsies, in which about twenty strains were usually studied from each animal, but one to three were taken from any one locality (e. g., fundus end of stomach), and often but one from any sample from a given region.

PLANT, SOIL, AND WATER STUDIES

An effort was first made to widen the range of known habitats. German bacteriologists (e. g., Puppel, 1912) speak very casually of "hay" and "fodder" as among the sources of the streptococci found in milk. Prescott (1906) reports streptococci as occurring on hay and grain (8 rye, 6 oats, 3 buckweat, and 1 wheat). Streptococci are known to be present in appreciable numbers in sewage-polluted water. Soils are also mentioned as habitats. Dr. V. A. Moore tells me that he frequently found streptococci in garden soil in Washington. The gardens, however, had been heavily and frequently manured. Besides this, there is very little definite information available. Andrewes (1906) states that, in nature, streptococci cannot grow and multiply for any length of time apart from the human body. Eighty-four samples of hay, grass, and leaves from country roadsides, pastures, park, and garden paths were examined. Streptococci were seen in but two cases, and three strains were isolated from the recently cut side of a hay stack. Soil and water from wood edges, moist roadside banks and brooks, woodroad humus, and near tie posts at mills, etc., were examined; of these eighteen samples, one sample of water (country roadside overflow) yielded a short-chained coccus organism (six to eight cocci in favorable media).

The other strains studied during the past four years were obtained from the following sources: (1) Human throats, chiefly diphtheria suspects; (2) alimentary canals (mouth to large intestine) of various animals—hen, pigeon, cat, and dog; (3) feces of cat, dog, man, cow, and horse; (4) milk, including a few mastitis suspects; and (5) blood (glanders and anthrax specimens), antisera, and pathologic conditions. A few pathogenic strains (fifteen) were obtained from other laboratories.

When I began my work in 1911, the types of streptococci found in milk had been sought for in human throats and in the feces of the

horse, the cow, and man. Bovine mouth streptococci are doubtless also important. And dogs, cats, and other small animals contribute also to the streptococcic flora of milk, even on many of the "inspected" farms. In the hope of confirming the diagnostic value of streptococcic reactions, 752 distinct strains were isolated and studied; and fifteen strains from other laboratories were compared with these. One hundred milk strains (Broadhurst, 1912) were studied in meat media for the Gordon reactions, and in litmus milk and neutral red broth (anaerobically); and 113 in meat extract media for the six Gordon reactions only (Table 14). The remaining 554 were studied more fully: first, for staining qualities or differences with stains—methylene blue, carbolfuchsin, Jenner's stain, Gram's stain, and Hiss's capsule stain; secondly, in the six Gordon media, as described (arabinose also tried, but dropped as evidently not of value), smears for microscopic examination being made from each tube just before titration; thirdly, in nine other media (Table 15), in the hope that one or more would be itself definitely of value, or aid by making distinctive complexes—for example, yielding certain Gordon combinations plus litmus milk changes, but without blood hemolysis, and without the power of growing at room temperature in gelatin. The other nine media used for these 567 strains included agar plates for colony characters; agar stabs plus paraffin oil for anaerobic growth; gelatin for liquefaction and the ability to grow at room temperature; plain broth varying in acidity, plus 0.5, plus 1.5, and plus calcium carbonate; a variation of Todd's neutral-red medium; blood agar (horse blood); and litmus milk. This made a total of five stains and fifteen media. Sugar-free broth and fermentation tubes of dextrose were also used for a small part of these, but discontinued later, as not of value. Observations of these media were made after a lapse of eighteen hours, twenty-four hours, two days, and three days; the litmus milk, gelatin, and broth observations were also examined after five days, and ten days, and the gelatin usually after fifteen days, also.

After these 554 strains were completed, it was decided to drop all routine stains except carbolfuchsin, and the Hiss capsule stain. Media observations were continued for litmus milk, blood agar, gelatin, plain broth (+0.5), agar plates, and the six Gordon media, the others* having proved of doubtful value. Some of the details for these 554

* Neutral-red has been discarded as not diagnostic by almost all workers. Todd (1910), and Crowe (1912-13) described distinctive colony characters on neutral-red agar. I tried 100 milks strains in neutral-red broth anaerobically and about 400 from other sources aerobically on two modifications of Todd's neutral-red agar without securing anything of differential value. Anaerobic conditions, fermentation tubes, and oil-covered agar stabs were also discontinued after our 400 strains had been studied.

TABLE 14
TITRATION RECORDS FOR 113 STRAINS OF STREPTOCOCCI TESTED IN MEAT EXTRACT MEDIA

MILK

Strain	Sample	Saccharose	Lactose	Salicin	Raffinose	Mannite	Inulin
1	1	2.2	2.2	2.8	0.2	2.0	1.9
2	2	1.2	1.5	0.4	1.2	0.0	0.5
3	3	—0.2	3.3	0.2	0.0	0.2	0.1
4	4	—0.1	1.2	0.2	—0.2	0.0	0.1
5	5	—0.3	0.1	0.0	—0.1	—0.2	—0.1
6	6	—0.2	0.2	—0.2	—0.1	—0.2	0.0
7	7	—0.2	3.0	—0.1	0.0	0.0	0.0
8	8	2.2	1.8	2.6	0.1	2.0	1.8
9	9	2.3	1.9	2.6	0.4	1.8	2.1
10	10	2.1	1.9	2.2	0.4	1.9	1.9
11	11	1.7	1.8	1.9	0.2	1.5	1.8
12	12	2.7	1.8	2.9	0.5	2.1	1.7
13	12	2.0	2.9	1.9	0.5	1.4	1.6

BOVINE FECES

1	1	3.6	3.5	3.4	0.4	0.0	0.1
2	2	2.9	3.3	3.2	3.3	0.0	2.2
3	3	3.0	3.2	3.0	3.2	0.0	2.6
4	4	2.9	2.6	3.5	3.1	0.0	3.1
5	5	2.8	3.2	3.2	3.1	0.1	3.0
6	6	2.6	2.9	3.7	3.6	0.2	0.3
7	6	2.7	3.0	3.9	3.2	0.2	0.4
8	7	1.8	3.3	4.2	0.4	2.2	0.3
9	8	2.6	3.5	4.1	0.4	2.4	0.2
10	8	2.5	3.5	4.2	0.5	2.3	0.2
11	9	3.1	2.7	2.9	3.1	0.1	3.6
12	10	2.8	2.8	2.8	3.4	—0.1	2.9
13	11	3.6	3.7	3.2	0.2	0.0	0.1
14	12	0.1	0.3	0.0	0.5	0.0	0.1
15	13	4.1	3.5	3.4	1.9	0.0	0.9
16	14	1.8	2.2	2.3	1.7	1.4	0.1
17	15	2.2	1.7	2.7	0.9	1.9	0.0
18	16	3.6	4.8	3.4	0.3	1.3	0.0
19	17	2.7	2.4	2.9	3.0	0.0	4.0
20	18	2.3	2.3	2.5	1.4	—0.1	3.9
21	18	2.4	2.5	3.3	2.8	0.0	4.1
22	19	3.4	2.8	3.8	0.2	2.2	0.1
23	20	0.1	0.0	—0.1	0.0	0.1	—0.2
24	21	0.0	—0.1	0.1	—0.1	0.1	—0.1
25	22	2.8	2.8	2.9	2.4	0.2	4.8
26	22	2.4	2.3	0.5	2.6	0.0	4.0
27	20	—0.1	—	—0.2	—0.1	0.0	—0.2
28	20	—0.2	—	—0.3	—0.1	—0.1	—
28	20	0.1	—	0.1	—0.1	—0.2	—
29	21	—0.2	—	—0.3	—0.2	—0.1	—
30	21	—0.1	—	—0.2	—0.2	—0.1	—

EQUINE FECES

1	1	0.3	0.0	1.0	0.1	0.2	0.2
2	2	2.6	2.8	3.2	0.2	1.5	0.2
3	3	2.8	2.9	3.5	0.5	1.7	0.1
4	4	0.9	0.5	0.6	—0.1	—0.2	0.0
5	5	1.9	1.8	2.0	1.5	—0.2	1.7
6	6	—0.1	0.1	—0.1	—0.1	—0.2	0.0
7	7	1.0	0.1	0.9	0.0	—0.2	—0.1
8	8	0.5	0.3	0.8	—0.2	—0.1	0.1
9	9	—0.2	0.2	1.3	—0.1	—0.4	0.1
10	9	2.6	2.9	3.0	1.4	1.5	0.2
11	10	1.5	0.4	2.0	0.2	0.2	0.2
12	11	1.5	2.0	0.9	1.5	1.6	0.0
13	12	1.8	2.2	1.9	1.4	1.5	0.8
14	13	1.4	0.2	0.5	0.0	—0.1	0.2
15	14	—0.1	0.3	0.2	0.0	0.1	0.1

TABLE 14—Continued
EQUINE FECES—Continued

Strain	Sample	Saccharose	Lactose	Salicin	Raffinose	Mannite	Inulin
16	14	3.3	3.5	3.4	1.3	2.1	0.3
17	15	3.4	2.7	3.2	0.5	2.0	0.1
18	16	3.4	3.3	3.3	0.4	1.9	0.2
19	16	3.5	3.5	3.4	1.5	2.0	0.2
20	17	0.9	0.1	1.0	0.0	—0.1	0.2
21	18	1.4	0.2	0.5	2.0	—0.1	0.2
22	18	—0.1	0.1	0.5	0.0	—0.2	0.1
23	19	4.8	5.3	0.0	4.9	0.8	3.4
24	20	2.0	2.6	2.4	2.1	0.0	1.3
25	21	1.8	2.4	2.5	1.5	1.8	1.1
26	22	2.9	0.1	3.7	2.4	—0.1	2.8
27	23	2.9	2.6	3.1	0.5	1.7	0.0
28	23	2.3	0.2	2.6	0.2	0.0	2.4
29	24	2.1	1.8	3.1	1.9	—0.1	2.8
30	24	2.5	2.5	1.7	2.0	—0.1	0.2
31	25	1.5	1.8	2.4	1.4	1.4	1.2
32	26	2.9	2.7	3.3	0.5	1.8	0.1
33	27	1.6	1.7	2.1	1.5	1.6	1.5
34	28	1.5	1.6	1.0	1.4	2.0	1.2
35	29	1.1	0.2	1.2	0.5	0.0	0.0
36	29	2.1	0.1	1.9	0.3	—0.1	2.6
37	30	1.6	1.8	1.2	1.6	1.6	1.4
38	30	1.5	2.2	1.4	1.4	1.6	1.4
39	31	2.8	1.6	3.1	2.6	0.0	3.2
40	32	1.0	1.9	2.6	0.9	1.7	0.9
41	33	1.5	1.9	1.0	1.1	1.7	1.7
42	34	3.3	0.2	2.7	2.3	0.0	1.7
43	34	0.1	2.6	4.2	0.2	0.1	0.2
44	35	1.7	1.3	2.4	0.5	0.0	2.6

HUMAN FECES

1	1	3.0	3.3	4.2	0.4	2.6	0.3
2	1	0.0	3.5	4.0	0.1	1.1	0.6
3	2	1.5	2.7	3.3	0.2	2.0	0.0
4	2	0.0	2.6	2.7	0.0	1.4	0.1
5	3	0.0	3.2	2.9	—0.1	0.0	0.0
6	4	4.0	2.8	3.5	0.2	0.0	0.2
7	5	2.2	1.4	3.6	0.2	0.3	0.0
8	6	0.0	3.0	3.1	0.1	0.0	0.1
9	7	3.8	2.6	2.7	0.1	0.0	0.1
10	8	2.2	0.5	3.9	0.3	1.6	0.3
11	9	2.7	3.1	3.1	0.0	1.5	0.3
12	9	3.5	3.2	3.2	0.0	2.2	0.4
13	9	3.1	3.5	2.5	0.1	1.9	0.0
14	10	0.1	2.6	3.3	0.0	1.2	0.1
15	10	0.0	2.6	3.3	—0.1	1.6	0.1
16	11	1.1	3.2	3.3	0.1	1.5	0.2
17	12	2.3	3.3	3.1	0.2	1.5	0.2
18	12	0.1	3.7	4.0	0.0	—0.1	0.1
19	13	4.0	2.7	4.0	0.3	1.1	0.0
20	14	3.2	2.6	2.9	0.2	1.7	0.1
21	14	0.2	2.3	3.4	0.1	2.0	0.0
22	15	0.2	2.0	2.0	0.2	0.3	0.0
23	16	3.4	2.9	3.2	0.1	1.8	0.0
24	17	1.4	1.9	1.6	1.1	1.6	1.4
25	17	1.6	2.5	3.4	0.3	1.2	0.2
26	18	2.7	3.4	3.7	0.4	2.5	0.0
27	19	0.1	2.5	3.1	—0.1	1.8	0.0
28	19	0.1	3.0	3.2	0.1	1.7	0.0
29	20	0.1	1.7	0.8	0.5	1.5	0.2
30	20	2.6	2.5	3.3	0.4	2.2	0.2
31	21	1.7	2.2	3.8	0.5	1.9	0.2
32	21	2.0	2.4	3.3	0.3	2.5	0.2
33	22	1.6	2.6	3.5	0.3	1.8	—0.1
34	22	2.2	2.2	3.5	0.4	1.2	0.2
35	23	3.4	3.0	2.7	0.2	2.2	—0.1
36	23	1.7	1.8	3.0	0.2	2.1	0.1
37	24	2.1	2.2	3.6	0.4	1.7	0.0
38	23	0.1	—	3.2	0.1	1.2	—0.1
39	25	2.6	2.6	0.1	2.4	0.2	0.5

strains are given in Table 15. Many records have been omitted as apparently not important; some of those given are of doubtful value, but they are included, as other investigators may find them confirmatory or otherwise useful.

From the work outlined above there has resulted a mass of details the comparison of which is an appalling task. Even after excluding the several special series (such as the meat and meat extract comparative series, and the constancy tests in Gordon media), there is for over 500 of the strains an average of eighty-five morphologic and physiologic records. To select the significant ones is very difficult. In the first place, it is almost impossible to bring such a mass of data into a form compact enough for a just consideration of the details leading to any real correlation of morphologic and physiologic characteristics. Secondly, morphologic terms are probably ultimately but an expression of physiologic powers and changes which are often unknown or unmeasurable. Distinctions based upon modifiable morphologic characters (such as the presence of a capsule and the chain length) are at best uncertain as morphologic distinction and may be very misleading in physiologic correlations. Thirdly, it is difficult to tell whether the sources from which the strains were isolated are themselves of classifying value, or whether they should be viewed only as "previous (and often temporary) conditions of servitude." Much study and many groupings and re-groupings of the data will be necessary to determine the importance (or the insignificance) of the sources from which the streptococci have been isolated. Several conclusions, however, may be drawn.

CONCLUSIONS

Streptococci occur less commonly in soils and water than most text-books imply. Hay, grass, etc., are apparently but temporary and accidental bearers of streptococci.

Streptococci are apparently as constantly a part of the flora of the mucosa and contents of the alimentary canal in cats, cows,* dogs, and horses as in man.

Streptococci vary greatly in the comparative ease† with which they may be isolated from various animal sources; for example, readily

* Kinyoun found streptococci in all but 2 of 2,000 calf autopsies. Andrewes (1906) reports them in the feces of fox, stoat, etc.

† Ease in isolation depends both on the relative number of streptococci and on the type of bacterial companions in that sample ("spreaders," etc.); but from repeated examinations of broth tubes of the samples it seems probable that the numeric relation is usually the most important factor. The optimistic statements of various workers regarding the dominance of streptococci in 18 to 36 hours are certainly not true of the streptococci of the wide range of habitats covered by this investigation.

TABLE 15
SELECTED DETAILS OF 538 STRAINS OF STREPTOCOCCI FROM VARIOUS SOURCES [MEAT MEDIA THROUGHOUT]
STRAINS FROM PLANTS

Strain	Long-est Chain Length	Com-plex Chain Length	Clearing in Broth (16-Day Period) Observation	Agar Colonies *	Visible Growth in Gelatin Days	Litmus Milk (10-Day Period) Observation	Colonies on Blood Agar (5-Day Period) Observation	Gordon Reactions					
								Saccharose	Lactose	Sulphur	Raffinose	Mannite	Inulin
25	10	12, 8	3	Usual	-1	No change	G - } haze	0.2	0.5	1.5	0.3	0.4	0.7
11	10	12, 8	3	Usual	-1	No change	G - } haze	0.1	0.2	4.2	0.1	0.6	0.1
18	8	2, 1	None	Usual	-1	A/2, C-10	—	3.1	3.0	3.0	2.1	2.6	2.1

STRAINS FROM WATER
(Strains from the same sample included in braces)

48	12	4, 2	10	Usual	-1	A, C-5	? Color	5.7	1.1	1.8	0.5	3.2	0.2
49	12	1, 2	10	Usual	1	A, C-5	? Color	5.5	4.0	1.8	0.5	3.2	0.2
50	12	4, 2	10	Usual	1	A, C-5	? Color	5.5	4.9	1.9	0.4	3.0	0.0
51	12	1, 2	10	Usual	1	A, C-6	? Color	1.7	4.6	1.9	0.6	3.3	—
52	12	1, 2	10	Usual	-1	A, C-5	? Color	5.5	4.6	5.2	0.4	3.0	0.3

STRAINS FROM MILK

7	12	1, 2	5	Usual, D	-1	A, C-1	Green	3.8	1.7	1.1	2.9	2.9	0.2
8	12	4, 2	5	Usual, D	-1	A, C-1	Green	3.6	1.1	1.5	2.5	3.5	0.3
9	10	2, 10	None	Usual, D	-1	A, C-1	Green	1.0	3.9	2.8	2.2	3.1	0.2
10	10	20, 8	None	Usual, D	-1	A, C-1	Green	0.9	0.2	0.8	0.5	0.1	0.1
11	10	12, 8	None	Usual, D	-1	A/2, C-1	G - } haze	0.0	0.2	0.1	0.0	0.2	0.0
12	10	12, 1	None	Concentric rings	-1	A, H ₂ O, C-1	Green	3.9	3.0	0.2	0.2	0.0	0.3
13	12	1, 2	None	Usual, D	-1	A, C-1	G - } haze	1.0	1.3	1.2	2.1	3.1	0.0
14	12	2	None	Usual, D	-1	A, H ₂ O, C-1	Green	3.0	3.7	4.1	3.2	2.8	0.4
15	8	2, 5	None	Usual, D	1	A, C-1	G - } haze	3.0	3.9	1.3	3.6	2.9	0.2
16	8	1, 3	None	Usual, D	1	A, C-1	Green	1.8	1.1	1.3	2.2	2.8	-0.2
17	15	3	None	Usual, D	-1	A, H ₂ O, C-1	Green	1.5	3.7	3.5	3.1	2.8	0.8
18	50	1	None	Usual, D	1	A, H ₂ O, C-1	Green	3.3	1.1	1.7	3.2	2.8	0.4
27	15	4, 5	None	Usual, D	-1	A, C-1	G - } haze	0.3	1.3	3.2	0.1	0.3	0.1
28	30	12, 8	None	Usual, D	1	A, C-1	G - } haze	3.3	3.9	0.1	0.2	0.1	0.1
29	25	2, 12	None	Muggy	None	No change	? Color	0.5	1.2	1.3	0.2	1.0	0.3
30	2	2	2	Usual	1	A, H ₂ O	Green	1.5	1.0	0.8	0.3	3.2	0.5
51	4	2	2	Usual	-1	A, H ₂ O	Green	1.8	1.7	1.8	0.3	3.2	0.2
55	6	2	2	Usual	1	A, H ₂ O	Green	1.1	4.1	5.0	0.5	3.2	0.3
60	40	5, 12	2	Usual	1	A, C-1	Green	0.1	3.0	-0.1	0.0	0.0	0.0
61	40	5, 12	2	Usual	-1	A, C-1	Green	0.2	1.0	0.1	-0.2	-0.1	-0.3

STRAINS FROM CAT THROAT

66	12	4, 8	None	Usual	2	A/2, C-2	Green	3.0	3.7	1.1	0.0	2.9	0.1
67	10	1, 8	None	Usual	2	A/2, C-2	Green	3.7	3.6	1.3	0.1	3.0	-0.1
68	10	1, 8	None	Usual	2	A/2, C-2	Green	3.5	3.8	1.2	-0.1	2.8	0.0
69	10	1, 8	None	Usual	2	A/2, C-2	Green	3.0	1.2	1.1	0.0	2.1	-0.1
70	12	1, 8	None	Usual	2	A/2, C-2	Green	3.0	3.7	4.3	0.0	3.0	0.0
71	10	1, 8	None	Usual	2	A/2, C-2	Green	3.1	3.1	3.5	0.1	2.8	0.1
72	10	4, 8	None	Usual	2	A/2, C-2	Green	3.3	3.5	3.7	0.0	2.8	0.2
85	10	1, 8	None	Usual	2	A/2, C-2	Green	1.2	3.1	3.7	0.1	2.8	0.0
86	10	1, 8	None	Usual	2	A/2, C-2	Green	3.7	3.7	1.1	0.0	3.0	0.2
87	10	2, 3	5	Usual	5	A/2, C-2	Green	1.2	4.6	2.8	2.7	0.1	1.2
88	12	1	None	Muggy	5	A/2, C-2	Green	1.1	4.0	3.0	2.5	0.1	1.1
89	12	1	None	Muggy	5	A/2, C-2	Green	2.1	2.5	0.2	0.0	1.8	0.0
89	12	1	None	Muggy	5	A/2, C-2	Green	2.1	2.6	0.1	0.0	2.0	0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	1.4	2.5	0.1	0.0	1.0	0.0
89	12	1	None	Muggy	5	A/2, C-2	Green	3.0	2.9	3.0	0.2	0.0	0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	3.0	2.7	3.2	0.1	0.0	0.2
89	12	1	None	Muggy	5	A/2, C-2	Green	3.7	1.0	1.7	0.2	3.1	0.2
89	12	1	None	Muggy	5	A/2, C-2	Green	1.8	1.0	1.1	0.3	2.6	0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	0.7	1.7	1.0	0.2	3.4	0.2
89	12	1	None	Muggy	5	A/2, C-2	Green	0.2	2.2	1.8	0.2	3.1	0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	1.1	2.8	1.2	3.4	0.2	1.0
89	12	1	None	Muggy	5	A/2, C-2	Green	1.2	3.0	1.1	3.4	0.3	1.7
89	12	1	None	Muggy	5	A/2, C-2	Green	2.5	2.8	3.9	1.7	0.0	2.3
89	12	1	None	Muggy	5	A/2, C-2	Green	2.8	2.5	2.3	0.2	2.0	2.7
89	12	1	None	Muggy	5	A/2, C-2	Green	2.6	2.3	2.1	0.3	2.0	2.7
89	12	1	None	Muggy	5	A/2, C-2	Green	2.3	2.3	1.7	0.1	1.7	2.7
89	12	1	None	Muggy	5	A/2, C-2	Green	2.8	3.9	3.7	0.0	3.2	0.3
89	12	1	None	Muggy	5	A/2, C-2	Green	1.2	2.9	2.9	4.3	4.0	1.0
89	12	1	None	Muggy	5	A/2, C-2	Green	2.5	2.4	1.0	0.2	2.5	0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	3.0	2.5	1.0	0.1	2.0	0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	2.3	2.7	2.0	2.3	0.0	3.1
89	12	1	None	Muggy	5	A/2, C-2	Green	2.6	2.6	2.0	0.0	2.0	-0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	2.6	2.8	2.8	2.1	-0.1	2.0
89	12	1	None	Muggy	5	A/2, C-2	Green	2.7	2.1	2.7	2.1	0.4	2.8
89	12	1	None	Muggy	5	A/2, C-2	Green	2.7	2.1	1.8	0.2	1.0	0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	2.7	2.7	1.9	0.1	1.8	0.0
89	12	1	None	Muggy	5	A/2, C-2	Green	2.7	2.7	2.0	0.2	2.1	0.2
89	12	1	None	Muggy	5	A/2, C-2	Green	2.4	2.7	1.7	0.2	1.9	0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	2.7	2.5	1.9	0.2	1.8	0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	2.8	3.0	2.0	0.1	2.2	0.2
89	12	1	None	Muggy	5	A/2, C-2	Green	2.9	2.8	2.7	2.1	-0.3	2.7
89	12	1	None	Muggy	5	A/2, C-2	Green	2.9	2.8	2.9	2.0	0.0	2.7
89	12	1	None	Muggy	5	A/2, C-2	Green	4.1	2.0	2.0	2.4	0.0	3.0
89	12	1	None	Muggy	5	A/2, C-2	Green	2.8	2.5	1.7	0.1	2.0	0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	3.1	2.5	2.7	2.4	0.0	-0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	3.0	2.8	3.0	2.1	0.1	5.7
89	12	1	None	Muggy	5	A/2, C-2	Green	2.9	2.8	2.8	2.7	-0.1	2.9
89	12	1	None	Muggy	5	A/2, C-2	Green	2.9	2.8	2.6	2.2	0.0	3.1
89	12	1	None	Muggy	5	A/2, C-2	Green	2.7	2.0	3.0	2.3	—	2.8
89	12	1	None	Muggy	5	A/2, C-2	Green	1.7	2.7	2.7	0.0	-0.1	0.1
89	12	1	None	Muggy	5	A/2, C-2	Green	2.5	2.4	2.7	0.2	0.1	0.1

STRAINS FROM CAT ESOPHAGUS AND STOMACH

1302	20	2, 6	Very slight	Muggy	None	A, D-1	Green	4.1	2.8	3.0	3.0	2.1	3.0
7	20	2, 0	Very slight	Muggy	None	A, C-1	Green	4.3	2.6	3.6	3.0	3.3	3.1
11	20	12, 20	Very slight	Fimbriate, pale	None	A, C-2	Green	2.5	2.3	2.4	2.6	0.0	2.5
14	20	20, 12	Very slight	Fimbriate, pale	None	A, C-2	Green	2.0	2.1	2.1	1.9	0.0	2.0
140	20	12, 20	Very slight	Fimbriate, pale	None	A, C-2	Green	2.5	2.1	2.5	2.1	0.0	2.6
7	16	2, 5	Very slight	Thin, irregular margin	None	A, C-2	Green - haze	2.1	2.2	1.1	0.1	1.9	0.1
8	16	2, 5	Very slight	Thin, irregular margin	None	A, C-2	Green	2.6	2.1	1.6	0.1	1.7	0.1
10	40	12, 20	Very slight	Fimbriate, pale	None	A, C-2	Green	2.6	2.3	2.1	2.1	-0.1	2.4
111	8	2	Very slight	Usual	None	A, C-1	No color - haze	1.1	2.5	3.3	3.0	3.3	2.9
3	8	2	Very slight	Flocculent, heavy	None	A, C-1	No color	3.0	2.5	3.8	2.0	2.6	3.0
12	8	2	Very slight	Flocculent	None	A, C-1	Green	1.2	2.8	3.5	2.7	2.9	3.0
14	8	2, 4	None	Usual	2	A/3, H ₂ O top, C-2	Green	4.1	2.7	6.1	0.0	3.5	0.2
15	10	2, 1	None	Usual	2	A/3, H ₂ O top, C-2	Green	0.2	3.1	6.1	-0.1	3.5	0.2
16	10	2, 4	None	Usual	2	A/3, H ₂ O top, C-2	Green	0.2	1.2	4.0	0.0	3.6	0.3
17	12	2, 1	None	Usual	2	A, C-1	Green	0.2	4.4	1.3	1.7	0.1	0.1
18	4	2, 4	None	Usual	2	A/3, H ₂ O top, C-2	Green	0.2	2.2	4.8	0.0	3.6	0.5
19	8	2, 1	None	Muggy	2	A/3, H ₂ O top, C-2	Green	0.2	2.6	5.0	0.0	3.6	0.2
50	8	2	None	Usual	15	A/3, H ₂ O top, C-2	Green	0.2	2.0	5.0	0.0	3.8	0.3
51	4	2	None	Usual	15	A/3, H ₂ O top, C-2	Green	0.2	2.3	1.0	0.0	-0.2	0.1
93	12	2, 4	5	Whiter	1	-A, a C	Green	5.0	3.1	1.1	0.2	0.1	0.1
94	12	2, 4	5	Usual	1	-A, a C	Green	1.8	3.8	1.1	0.2	0.0	0.0
12	25	5	None	Usual	1	A/2, C-1	Green	0.1	1.5	1.2	0.2	-0.1	-0.2
13	20	5, 12	None	Usual	1	A/2, C-1	Green	0.0	4.5	1.3	-0.2	-0.1	-0.1
11	20	20, 12	None	Usual	1	A/3, C-1	Green	0.1	3.7	4.0	0.2	0.1	0.0
41	8	2, 4	None	Usual	2	-A, C-2	Green	1.5	3.7	4.7	0.3	0.0	0.0
42	8	2, 1	None	Usual	2	-A, C-2	Green	1.2	3.7	1.1	0.0	-0.1	0.2
13	8	2, 1	None	Usual	2	-A, C-2	Green	1.2	1.2	5.5	0.0	0.0	4.2
22	10	2, 12	3	Usual	1	No change	Green	-0.1	0.0	0.1	0.0	-0.1	2.0
33	10	5, 20	3	Usual	1	No change	Green	2.2	2.0	-0.2	0.0	0.0	0.0
151	60	20, 16	7.2	Muggy	None	A, C-2	G - haze	2.7	2.0	2.7	0.0	0.0	0.1
152	60	20, 16	1.2	Usual	None	A, C-2	G - haze	2.7	2.5	2.7	0.1	0.0	0.1

1	12	8	None	Thin, irregular margin.	1	A, C, 1	Green	0.1	3.6	1.0	-0.2	-0.1	-0.3
2	12	8	None	Thin, irregular margin.	1	A, C, 1	Green	0.1	1.6	1.2	-0.2	-0.1	-0.3
3	20	8	None	Thin, irregular margin.	1	A, C, 1	Green	-0.2	4.3	1.0	-0.2	0.0	0.0
4	25	12	None	Usual	1	A, C, 1	Green	0.0	4.4	4.7	-0.2	-0.2	-0.1
5	100	8, 12	None	Usual	1	A, C, 1	Green	-0.1	4.7	4.0	-0.1	-0.1	-0.1
6	25	1, 12	None	Usual	-1	A, C, 1	Green	0.0	1.4	3.9	-0.2	0.0	-0.1
7	15	8, 12	None	Usual	-1	A, C, 1	Green	0.0	1.0	1.0	-0.2	-0.1	-0.1
8	15	1	None	Muggy	2, thin	A, C, 1	Green	0.3	5.2	3.5	3.3	-0.1	1.0
9	20	1, 12	None	Muggy	1	A, C, 1	Green	0.1	1.0	1.1	-0.2	-0.1	-0.2
10	20	8, 12	None	Usual	1	A, C, 1	Green	0.2	1.5	3.9	0.1	-0.1	-0.1
11	20	8, 12	None	Usual	1	A, C, 1	Green	-0.2	1.1	1.2	0.1	0.1	0.5
15	20	12, 100	None	Usual, D.	1	A, C, 1	Green	0.0	1.0	1.1	-0.2	-0.2	-0.1
16	100	20, 8	None	Usual	1	A, C, 1	Green	0.0	1.8	1.0	0.2	-0.1	-0.1
17	20	8, 12	None	Usual	1	A, C, 1	Green	0.0	4.1	3.9	-0.2	0.1	-0.1
18	15	8	None	Usual	1	A, C, 1	Green	0.0	4.7	3.8	0.0	0.1	0.0
19	100	50, 12	None	Usual	1	A, C, 1	Green	0.1	1.3	1.2	-0.2	-0.2	-0.2
20	200	1, 12	None	Usual	1	A, C, 1	Green	0.3	1.9	3.0	0.0	0.2	0.0
21	15	2, 4	None	Usual	2	A, C, 1	Green	1.3	3.6	1.7	0.2	0.2	0.3
22	8	2, 4	None	Usual	2	A, C, 1	Green	1.3	3.6	1.6	0.0	0.0	-0.2
23	8	2, 4	None	Usual	2	A, C, 1	Green	1.5	3.8	5.0	0.1	0.2	0.1
24	0	2, 1	None	Usual	2	A, C, 1	Green	2.3	3.0	1.5	0.0	0.1	0.0
25	0	2, 4	None	Usual	2	A, C, 1	Green	0.1	3.7	1.0	0.0	0.1	0.2
26	0	2, 4	None	Usual	2	A, C, 1	Green	1.3	3.7	1.0	0.1	0.1	0.2
27	0	2, 1	None	Usual	2	A, C, 1	Green	1.5	3.9	5.2	0.1	-0.1	0.0
28	0	2, 1	None	Usual	2	A, C, 1	Green	1.2	1.0	4.0	0.0	0.1	0.1
29	0	2, 1	None	Usual	2	A, C, 1	Green	1.5	3.8	4.1	0.0	0.0	-0.1
30	0	2, 4	None	Usual	2	A, C, 1	Green	1.2	3.7	5.3	0.3	0.0	0.2
31	0	2, 1	None	Usual	2	A, C, 1	Green	4.0	3.6	4.5	0.1	0.0	0.0
32	0	2, 4	None	Usual	2	A, C, 1	Green	4.7	3.7	4.6	-0.1	0.1	0.0
33	0	2, 4	None	Usual	2	A, C, 1	Green	1.3	1.6	4.6	0.0	0.1	0.0
34	0	2, 1	None	Usual	2	A, C, 1	Green	1.5	3.8	5.1	0.0	-0.1	0.1
35	0	2, 1	None	Usual	2	A, C, 1	Green	1.3	3.5	4.1	0.0	0.1	0.0
36	0	2, 1	None	Usual	2	A, C, 1	Green	1.1	1.1	4.1	0.0	-0.1	0.2
37	0	2, 1	None	Usual	2	A, C, 1	Green	1.6	1.1	6.3	0.1	-0.1	0.2
38	0	2, 1	None	Usual	2	A, C, 1	Green	4.6	1.2	1.9	0.0	0.0	0.1
39	0	2, 1	None	Usual	2	A, C, 1	Green	1.0	1.4	5.0	0.1	0.0	0.3
40	0	2, 1	None	Usual	2	A, C, 1	Green	0.1	2.5	5.7	0.0	0.0	0.2
41	0	2, 1	None	Muggy	2	A/2, H ₂ O, C, 2	Green	0.1	2.1	5.0	0.0	0.0	0.1
42	0	2, 1	None	Muggy	2	A/2, H ₂ O, C, 2	Green	0.2	2.5	1.8	0.1	3.6	0.1
43	0	2, 1	None	Muggy	2	A/2, H ₂ O, C, 2	Green	0.0	2.3	5.3	0.0	3.4	0.2
44	0	2, 1	None	Usual	2	A/2, H ₂ O, C, 2	Green	0.2	3.1	3.7	0.1	1.1	0.1
45	0	2, 1	None	Usual	2	A/2, H ₂ O, C, 2	Green	0.0	2.6	0.0	-0.1	3.6	0.2
46	0	2, 1	None	Usual	2	A/2, H ₂ O, C, 2	Green	0.3	2.9	6.0	0.0	3.4	0.2
47	0	2, 1	None	Usual	2	A/2, H ₂ O, C, 2	Green	0.1	2.0	4.7	0.0	3.3	0.1
48	0	2, 1	None	Usual	2	A/2, H ₂ O, C, 2	Green	0.1	2.1	5.4	0.0	3.7	0.1
49	0	2, 1	None	Usual	2	A/2, H ₂ O, C, 2	Green	0.2	1.5	1.8	0.1	3.6	0.2
50	0	2, 1	None	Harder, thin	2	A/2, H ₂ O, C, 2	Green	0.0	1.7	5.2	0.2	3.6	0.2
51	0	2, 1	None	Harder, thin	2	A/2, H ₂ O, C, 2	Green	0.3	1.8	1.0	0.0	3.4	0.1
52	0	2, 1	None	Usual	3	A, C, 2	Green	1.1	3.3	3.9	0.0	-0.1	0.0
53	0	2, 1	None	Usual	3	A, C, 2	Green	4.3	3.8	1.1	-0.1	0.1	0.1
54	0	2, 1	None	Usual	1	A, C, 2	G -> haze	0.2	2.2	1.5	0.2	3.2	0.2
55	0	2, 1	None	Usual	2	A, C, 2	Green	0.2	1.0	4.5	-0.1	2.7	0.2
56	0	2, 1	None	Usual	2	A, C, 2	Green	1.4	3.0	0.0	3.7	0.0	0.4
57	0	2, 1	None	Usual	1	A, C, 2	G -> haze	0.2	2.3	1.5	0.1	2.8	0.2

STRAINS FROM FAT FECES

1	30	8, 12	3-5	Usual	-1	A, C, 1	Green	2.1	2.7	3.0	0.1	2.0	0.3
2	30	8, 1	3-5 (1)	Usual	1	A, C, 1	Green	2.6	2.9	3.0	0.1	1.7	0.1
3	0	12	12, 4	Usual to yellow white	1	A, C, 1	Green	4.7	3.9	4.5	0.0	0.1	0.2
4	0	12	1, 8	Usual	2	A, C, 1	Green	0.0	4.2	1.1	0.2	0.2	0.2
5	0	12	1, 8	Usual	2	A/2, C, 2	Green	4.6	3.8	4.7	0.1	0.0	0.1
6	0	12	2, 1	Usual, irregular margin	2	A/2, C, 2	Green	1.2	3.0	4.2	0.0	0.1	0.0
7	0	12	2, 1	Usual	2	A/2, C, 2	Green	1.3	3.9	1.1	0.1	0.1	0.0
8	0	12	2, 4	Hard	2	A/2, C, 2	Green	4.1	3.9	3.7	0.1	-0.1	0.2
9	0	12	2, 4	Usual	2	A/2, C, 2	Green	4.6	3.8	4.7	0.0	0.0	0.0
10	0	12	2, 1	Usual	2	A/2, C, 2	Green	4.4	3.7	4.1	0.2	0.0	0.0
11	0	12	2, 1	Usual	2	A/2, C, 2	Green	4.3	3.0	1.1	0.0	-0.1	-0.1

STRAINS FROM HOG THROAT

1	8	1, 2	None	Concentric rings, muggy ?	1	A, 0, C	Green ?	1.7	1.1	8.0	0.5	4.0	0.2
2	8	1, 2	None	Muggy	1	-A, 0, C	Green ?	1.5	1.4	6.0	0.5	4.2	0.2
3	12	4	None	Muggy	1	-A, 0, C	Green ?	4.7	1.0	5.9	0.5	1.2	0.1
4	10	1, 8	None	Muggy	1	-A, 0, C	Green ?	4.7	1.5	5.8	0.0	1.0	0.4
5	10	4, 8	None	Muggy	1	A, 0, C	Green ?	4.5	1.3	6.0	0.6	4.0	0.2
6	10	8, 15	None	Muggy ?	1	A, C, 5	Green	5.3	1.9	4.2	0.3	0.0	0.2
7	10	12, 20	None	Usual	1	A/2, C, 5	No color	0.1	3.2	3.5	0.2	1.1	0.3
8	10	8, 12	None	Usual -> heavier	-1	A, 2, C, 5	G-brown	1.1	1.2	3.5	0.2	1.1	0.1
9	10	8, 12	None	Usual -> heavier	-1	A, C, 2	No color	3.8	3.4	3.8	0.1	3.2	0.2
10	25	2, 8	None	Usual	-1	A, C, 2	Green	3.8	3.8	3.0	0.2	3.0	0.0
11	30	4, 12	None	Usual	-1	A, C, 2	Hemolysis	2.7	3.9	3.9	0.2	3.0	0.1
12	8	1, 12	None	Usual	-1	A, C, 2	Hemolysis	0.0	1.2	1.7	0.3	2.7	0.0
13	12	4, 12	None	Usual	-1	A/2, C, 2	Hemolysis	0.2	1.1	1.0	0.2	2.9	0.0
14	16	2, 4	None	Usual	-1	A, H ₂ O, C, 2	Hemolysis	0.2	1.1	4.5	0.3	4.0	0.0
15	16	2, 1	None	Usual -> irregular margin	-1	A, C, 5	Green	0.0	3.1	4.3	0.1	0.1	0.0
16	16	2, 1	None	Usual -> irregular margin	-1	A/2, C, 5	Green	4.1	3.7	4.1	0.5	0.1	0.0
17	16	4, 8	None	Usual	-1	A, C, 0	Green	3.0	3.2	3.0	0.7	2.5	0.2
18	8	2, 4	None	Usual	-1	-A, 0, C	Green	3.2	3.1	3.1	0.7	2.5	0.2
19	10	1, 8	None	Usual	1	-A, 0, C	No color	3.1	3.1	3.1	0.5	2.8	0.2
20	10	2, 1	None	Usual -> heavier	-1	A/2, C, 2	No color	0.3	3.8	1.1	0.2	2.7	0.0
21	8	2, 4	None	Usual -> heavier	1	A/2, C, 2	Green	0.1	3.0	4.5	0.1	3.0	0.0
22	8	2, 4	None	Usual	None	-A, 0, C	No color	3.9	2.2	3.1	3.1	2.7	0.0
23	20	2, 8	None	Usual	None	A, 0, C	No color	3.0	2.2	3.1	1.1	2.7	0.0
24	12	2, 1	None	Usual	None	A, 0, 1	Green	3.0	1.9	3.0	1.2	2.6	0.0
25	12	2, 8	None	Usual	None	-A, 0, C	Green	2.9	2.2	3.0	1.1	2.5	0.1
26	12	8, 4	None	Usual	None	-A, 0, C	Green	3.1	2.1	3.0	0.0	2.6	0.0
27	10	8, 4	None	Usual	None	A, 0, 0	Green	3.0	2.1	3.0	0.9	2.1	0.0
28	10	12, 8	None	Usual	1	-A, 0, C	G -> haze	0.2	0.1	2.5	0.2	1.8	0.1
29	10	12, 8	None	Usual -> heavy margin	1	A, 0, C	G -> haze	0.2	0.1	2.5	0.2	1.0	0.2
30	8	2, 4	None	Usual	1	A/2, C	G -> haze	2.8	2.7	3.2	2.2	2.4	2.1
31	8	2, 4	None	Usual	1	A/2, C	No color	2.7	2.7	3.3	2.3	2.1	2.1
32	8	2, 4	None	Muggy	Liq. 1	A/2, C	Green	3.1	2.9	3.0	0.1	2.6	0.1
33	8	2, 4	None	Muggy	Liq. 1	A/2, C	Green	3.7	2.9	2.9	0.1	2.7	0.1
34	20	1, 8	None	Heavier margin	2	A, 0, C	G -> haze	0.1	2.0	2.5	0.1	1.8	0.0
35	20	1, 8	None	Heavier margin	2	A, 0, C	G-brown	0.2	1.8	2.0	0.1	1.8	0.1
36	8	2, 1	None	Muggy	Liq. -3	A/2, C	No color ?	3.0	2.9	3.1	0.2	2.7	0.1
37	8	2, 1	None	Usual	Liq. -3	A/2, C	No color ?	4.2	3.1	3.5	0.1	2.6	0.0
38	8	2, 4	None	Muggy	Liq. -1	A/2, C	No color ?	2.8	2.9	3.5	0.3	2.5	0.1
39	8	2, 4	None	Muggy	Liq. 1	A/2, C	G -> haze	2.9	2.9	3.2	0.1	2.7	0.1
40	20	4, 8	None	Heavy rim	2	-A, 0, C	Green	1.1	1.8	2.9	0.1	1.0	0.1
41	30	8, 12	None	Muggy	3	A, 0, C	G -> hemolysis	0.3	2.1	2.6	0.2	1.8	0.1
42	40	8, 20	None	Heavy rim	3	A, 0, C	Green	1.4	2.2	2.2	0.2	1.9	0.2
43	12	2, 8	None	Heavy rim	3	A, 0, C	Color	2.5	2.1	3.2	1.0	2.1	1.0
44	8	2, 1	None	Muggy	Liq. 1	A/2, C, 1	Color	1.2	2.9	3.6	0.3	3	3.1
45	8	2, 1	None	Usual	1	A/2, C, 1	Green	2.8	2.9	2.9	0.2	2.7	2.2
46	8	2	None	Very pale	1	A/2, C, 1	Green	3.0	2.9	2.9	0.3	2.7	2.5
47	8	2, 1	None	Usual	1	A/2, C, 3	Green	3.0	2.7	2.7	2.2	2.1	2.1
48	10	8, 12	None	Thin, irregular margin	5	A, C, 3, 5	Green	2.2	1.7	1.1	1.0	1.9	2.5
49	10	8, 12	None	Thin, irregular margin	5	A, C, 3, 5	Green	2.2	1.8	1.4	1.5	1.8	2.5
50	10	12, 8	None	Thin, irregular margin	10	A, 0, C	G -> haze	0.2	1.7	1.4	1.3	1.7	2.5
51	20	2, 8	None	Usual -> muggy	1	A, C, 2	G -> haze	2.1	3.0	3.1	0.2	2.8	0.2
52	50	8, 12	None	Flocculent	2	A, 0, C	G -> haze	0.0	0.1	2.5	0.1	2.0	0.1
53	20	8, 2	None	Flocculent	-1	A, 0, C	G -> haze	0.5	0.2	2.8	0.5	2.0	0.1
54	20	4, 12	None	Usual	5	A, 0, C	No color	2.4	1.7	1.0	2.0	1.0	2.0
55	12	4, 8	None	Usual	5	A, 0, C	No color	2.3	1.9	1.0	1.0	1.8	2.0
56	8	2, 1	None	Muggy	-1 Liq	-A, H ₂ O, C, 1	Green	3.0	3.2	3.2	0.2	3.2	0.1
57	8	2, 1	None	Usual	15	A, 0, C	Green	2.3	1.8	1.5	1.1	1.7	2.5
58	10	1, 8	None	Usual	15	A, 0, C	Green	2.1	1.8	1.0	1.7	2.4	2.7
59	10	5	None	Usual	15	A, 0, C	Green	1.9	1.6	1.5	1.8	2.1	2.3
60	40	8, 12	None	Usual	5	A, 0, C	Green	2.3	1.6	1.6	1.0	1.9	2.6
61	10	8, 12	None	Usual	15	A, 0, C	Green	2.3	1.4	1.7	1.5	1.8	2.6
62	40	12, 8	None	Usual	15	A, 0, C	G -> haze	2.6	2.5	2.0	2.1	2.1	1.7
63	100	10, 12	None	Concentric rings, thin marg.	5	A, 1, 1	Hemolysis	3.0	2.7	2.7	0.3	0.7	-0.1
64	80	12, 8	None	Usual	10	A, H ₂ O, C, 2	Hemolysis	2.0	2.8	2.1	0.1	0.1	-0.1
65	100	12, 8	None	Usual	10	A, H ₂ O, C, 2	Hemolysis	2.8	2.9	2.1	0.2	0.0	0.0
66	200	8, 20	None	Usual	10	A, H ₂ O, C, 2	Hemolysis	2.0	2.8	2.8	0.2	0.2	0.1
67	200	10, 20	None	Usual, irregular margin	16	-A, 0, C	G -> Hemolysis	3.0	2.6	2.9	2.0	2.1	1.1
68	200	10, 60	None	Usual	3	A, H ₂ O, C-1	G -> Hemolysis	3.2	2.7	2.9	2.0	2.3	1.0
69	200	20, 60	Align. clear	Flocculent	None	-A, 0, C	Pale	2.6	1.0	0.0	0.1	0.2	0.1
70	200	40, 20	None	Usual	10	A, C, 3	Hemolysis	3.0	2.1	2.1	2.3	0.0	-0.1
71	200	8, 12	None	Usual	None	A, C, 3	G -> haze	2.7	2.6	1.6	0.1	1.7	0.0
72	80	20, 8	None	Usual	3	A, C, 3	Hemolysis	3.0	2.7	2.2	-0.1	0.1	0.0
73	20	20, 12	None	Usual	3	A, C, 3	Hemolysis	2.7	2.7	2.7	0.0	0.1	0.0
74	12	2, 8	None	Usual	4	A, C, 2	No color	5.6	5.2	5.8	4.8	0.0	0.0
75	40	5	5-10	Usual	None	A, 0, C	Color	1.2	3.5	3.2	0.2	-0.1	0.1
76	70	20, 8	5-10	Usual	None	-A, 0, C	Color	1.5	3.1	3.3	0.3	0.0	0.0
77	12	20, 8	5-10	Usual -> muggy	1	A, C, 3	Green	4.0	3.8	3.7	3.1	2.9	0.9
78	30	20, 8	5-10	Usual	None	A, C, 3	Green	3.5	4.0	3.7	2.8	2.8	0.9
79	50	20, 8	5-10	Usual	None	No change	No color	0.2	0.1	0.1	-0.1	-0.1	-0.1
80	50	1, 8	None	Usual	None	A, 0, 3	No color	4.7	4.0	4.0	0.2	0.0	0.0

TABLE 15—Continued

Strain	Longest Chain Length Seen	Common Chain Lengths	Clearing in Broth (10-Day Observation), Days	Agar Colonies *	Visible Growth in Gelatin Days	Litmus Milk † (10-Day Period Observation)	Colonies ‡ on Blood Agar (3-Day Period Observation)	Gordon Reactions					
								Saccharose	Lactose	Starch	Raffinose	Mannite	Inulin
STRAINS FROM DOG ESOPHAGUS AND STOMACH													
273	0	2	—1	Usual	None	Alk 7, O C	Haze	0.1	—0.2	—0.1	0.0	0.0	0.3
271	6	2	—1	Usual	None	Alk 7, O C	Haze	0.2	0.1	0.1	0.1	0.1	—0.2
275	6	2	—1	Usual	None	Alk 7, O C	Haze	0.0	0.1	—0.1	0.1	—0.1	0.0
276	12	8, 1	10	Usual → flocculent	2	A, O C	White	0.0	1.2	0.0	0.0	0.0	0.0
277	20	2, 12	10	Usual → mucgy.	2	A/2, 1:10	White	0.0	4.4	0.0	0.1	—0.1	0.1
278	12	2, 4	10	Usual → mucgy.	2	A/2, C 10	G-brown	0.1	4.3	0.1	—0.1	0.0	0.1
279	30	4, 12	10	Usual → mucgy.	2	A, C 10	G → Hemolysis	0.0	1.2	—0.1	0.0	0.0	0.1
280	500	20, 12	5	Usual	None	Alk 7, O C	Green	1.7	1.0	—0.1	0.1	0.0	0.0
281	8	2, 1	None	Usual	1 Liq.	A, O —1	G → haze	3.5	3.0	1.1	0.1	3.5	0.1
1	30	4, 8	10	Concentric rings, mucgy	1	—A, 1:10?	Green	5.7	5.1	1.7	0.2	2.2	0.0
2	25	4, 8	None	Concentric rings, mucgy	1	—A, C 10?	No color	5.5	5.4	4.7	0.3	1.3	0.1
3	12	8	5	Mucgy, concentric rings	1	—A, O C	No color	5.8	5.1	1.1	0.3	2.2	0.2
1	150	8, 20	10	Mucgy, concentric rings	1	—A, C 10	No color	0.0	5.1	4.7	0.1	0.6	—0.1
5	150	8, 20	5	Mucgy, concentric rings	2	A, C 5	No color	0.0	5.1	1.1	0.1	0.1	0.0
6	500	1, 20	5	Mucgy, concentric rings	1	A, C 5	No color	0.0	5.0	4.3	0.3	0.3	0.2
13	12	2	None	Mucgy ?	1	A, C 5	G → haze	0.2	1.3	4.1	1.5	0.1	0.0
14	12	4	10	Mucgy	2	A, C 5	G → haze	0.0	1.1	4.4	0.5	—0.1	0.0
15	10	2, 8	1	Mucgy	2	A, C 5	G → haze	0.1	4.3	1.2	0.8	0.0	0.1
200	20	8, 12	10	Tiny	5	A, O, C	Green	2.1	1.0	1.0	1.2	2.0	2.4
201	40	8, 12	10	Tiny	5	A, O, C	G → haze	2.2	1.7	1.7	1.5	2.1	2.7
202	10	8, 12	10	Tiny	5	A, O, C	G → haze	2.3	1.8	1.8	1.4	1.9	2.6
203	10	8, 12	10	Tiny	5	A, O, C	G → haze	2.3	1.7	1.8	1.8	1.9	2.6
204	10	8, 12	10	Tiny	5	A, O, C	G → haze	2.2	1.6	1.7	1.5	1.9	2.6
205	10	8, 12	10	Tiny	5	A, O, C	G → haze	2.2	1.5	1.7	1.5	1.9	2.6
206	10	8, 12	10	Tiny	5	A, O, C	G → haze	2.2	1.0	1.7	1.5	2.1	2.6
207	10	8, 12	10	Tiny	5	A, O, C	G → haze	1.9	2.0	1.8	1.2	1.8	2.6
208	10	8, 12	10	Tiny	5	A, O, C	G → haze	2.4	1.5	1.7	1.0	1.8	2.6
209	10	8, 12	10	Tiny	5	A, O, C	G → haze	2.2	1.9	1.1	1.8	2.0	2.6
210	10	8, 12	10	Tiny	5	A, O, C	G → haze	2.5	1.9	1.8	1.3	2.0	2.7
211	10	8, 12	10	Tiny	5	A, O, C	G → haze	2.5	1.0	1.7	1.3	2.0	2.6
212	10	8, 12	10	Tiny	5	A, O, C	G → haze	2.4	1.5	1.7	1.1	1.7	2.6
213	10	12, 1	10	Usual, mucgy	1	A/2, H ₂ O, C —1	G → haze	3.7	3.1	3.0	0.2	1.1	0.0
214	10	12, 1	10	Usual, mucgy	—1	A/2, H ₂ O, C —1	G → Hemolysis	3.1	3.6	3.0	0.0	0.1	0.1
215	10	12, 1	10	Usual → irregular margin, concentric rings	3	A, C 2	Hemolysis	3.2	3.1	2.8	0.1	0.0	0.2
216	100	20, 10	10	Usual → irregular margin, concentric rings	3	A, C 2	Hemolysis	3.2	3.1	2.6	0.1	0.0	0.0
217	100	8, 40	10	Usual → irregular margin, concentric rings	2	A, U 2	Hemolysis	3.2	3.0	2.8	—0.1	0.0	0.0
218	12	2, 4	None	Usual → mucgy	—1 Liq.	A, H ₂ O, C —1	G → haze	3.7	3.5	3.8	0.0	3.2	—0.1
219	300	10, 80	—1	Usual	2	A, C 2	Hemolysis	2.9	2.5	2.7	0.0	0.1	0.1
220	300	40, 80	—1	Usual	10	A, C 2	Hemolysis	3.1	2.6	—0.1	0.3	0.0	0.0
221	300	40, 80	—1	Usual	2	A, O 2	Hemolysis	3.0	2.5	2.2	0.1	0.0	—0.1
222	500	4, 20	Alkal. clear	Flocculent	None	A, O, C	G → haze	1.8	0.0	—0.1	0.0	0.0	0.2

STRAINS FROM DOG INTESTINE

16	30	1, 5	10	Concentric rings, mucgy.	2	—A, O, C	No color	—0.1	5.5	3.0	0.1	0.7	0.1
17	30	1, 5	5	Concentric rings, mucgy.	2	A, C 5	Green	0.0	5.2	4.0	0.1	0.1	0.1
18	10	2, 8	10	Concentric rings, mucgy.	1	0, yellow	No color	4.6	1.1	6.0	0.5	1.7	0.5
19	12	2, 5	None	Concentric rings, mucgy.	1	—A, C 10	Green	4.9	1.3	5.7	0.1	3.0	0.3
20	8	2, 8	None	Mucgy	1	—A, C 10	Green	4.7	1.2	5.8	0.1	1.0	0.3
21	10	12, 1	10	Mucgy, concentric rings	2	A, C 5	No color	0.0	5.5	4.1	0.2	0.0	0.2
22	30	8, 20	2	Mucgy, concentric rings	2	A, C 5	Green	0.1	5.7	3.9	0.0	0.2	0.2
189	80	8, 20	None	Usual	—1	A, O 1	Green	0.2	3.2	2.8	0.1	1.1	0.2
190	80	8, 20	10	Usual	2	A, O, C	Green	0.1	3.6	2.9	0.1	1.7	0.1
191	40	2, 8	None	Usual	3	A, O, C	Green	0.1	3.5	2.9	0.0	0.0	0.2
223	100	10, 20	2	Concentric rings, thin marg.	15	A, C 1	Hemolysis	3.0	2.8	2.6	0.1	0.1	0.0
224	100	40, 20	2	Concentric rings, thin marg.	15	A, C 2	Hemolysis	3.1	2.9	2.9	0.3	—0.1	0.2
225	8	2, 1	5	Usual	2	A/2, C 5	No color	2.9	2.8	2.8	0.0	0.0	0.1
226	8	2, 1	5	Concentric rings, thin marg.	2	A, C 1	Hemolysis	2.9	2.8	2.7	0.1	0.1	0.0
227	10	20, 12	1	Concentric rings, thin marg.	15	A, C 1	Hemolysis	3.0	2.1	2.7	0.1	0.1	0.0
228	8	2, 1	5	Concentric rings, thin marg.	15	A, C 2	Hemolysis	3.1	2.7	2.7	0.2	0.1	0.0
229	8	2, 1	5	Mucgy ?	2	A/2, C 5	Green	4.4	2.5	3.9	2.3	—0.1	0.0
230	8	2, 1	5	Mucgy ?	2	A/2, C 5	Green	1.7	2.9	3.7	2.5	0.2	0.0
231	12	2, 4	10	Usual	3	A, C 2	1 Color → yellow	4.9	4.5	1.3	0.2	2.0	0.0
232	8	2, 4	None	Usual	3	A/2, 1:2	Green	2.5	4.0	1.1	0.4	2.0	0.1
233	100	20, 30	None	Usual	None	A/2, C 2	Hemolysis	3.0	2.6	2.6	0.2	0.2	0.1
234	200	12, 40	5	Usual	10	A/2, C 2	Hemolysis	2.9	2.9	2.5	0.2	0.0	0.1
235	200	12, 8, 40	5	Usual	10	A/2, C 2	Hemolysis	2.9	2.5	2.1	0.2	0.0	0.0
236	200	12, 8, 40	5	Usual	10	A/2, C 2	Hemolysis	2.8	2.9	2.5	0.2	0.1	0.1
237	80	2, 1	5	Thin, irregular margin	None	A, C 5	Green	5.2	5.3	5.9	4.0	0.1	1.3
238	80	2, 1	5	Thin, irregular margin	None	A, C 3	Green	5.1	5.0	4.1	0.1	1.2	0.1
239	8	2, 9	10	Usual → mucgy	1	A, O 1	G → haze	0.1	3.1	1.7	—0.1	0.1	0.1
240	12	2, 2	10	Usual	1	A, O, C	G → haze	0.1	3.3	1.6	—0.1	0.0	0.0
241	8	2	3	Usual	—1	A, O, C	G → haze	4.6	4.7	1.5	0.0	0.0	0.0
242	100	20, 10	10	Usual → mucgy	5	A, C 2	Hemolysis	3.2	3.0	2.8	0.0	0.1	0.0
243	100	20, 10	10	Usual → mucgy	2	A, C 2	Hemolysis	3.3	3.0	0.2	0.2	0.0	0.0
244	50	8, 12	None	Usual → mucgy	—1	A, O, C	G → haze	0.0	3.8	3.0	0.0	1.1	—0.1
245	12	2, 1	None	Mucgy	—1	A, H ₂ O, C —1	G → haze	3.8	3.8	1.0	0.1	2.9	0.1
246	8	2	None	Mucgy	—1	A, H ₂ O, C —1	G → haze	3.6	3.7	4.0	0.1	3.5	—0.1
247	20	2, 8	—1	Usual ?	2	A, C 2	G → haze	5.1	5.5	5.2	4.9	0.5	1.8
248	10	2, 8	None	Mucgy	—1	A, H ₂ O, C —1	No color → haze	2.8	3.2	3.6	0.1	3.2	—0.1
249	8	2, 1	None	Mucgy	—1	A, H ₂ O, C —1	No color → haze	3.6	3.3	3.7	0.2	3.1	0.1

STRAINS FROM DOG FECES

75	20	12	2	Mucgy ?	2	A, H ₂ O, C 2	Green	5.4	1.0	3.0	0.4	3.0	0.1
76	80	8, 12	2	Mucgy ?	2	A/2, C 5	Green	0.1	5.3	1.3	0.2	1.3	0.1
77	40	8, 12	2	Usual	2	A	Green	0.1	5.2	1.1	0.1	1.2	0.0
78	30	12, 4	2	Usual, D	2	—A, O 1	Whitish	4.3	3.0	3.5	0.3	0.1	0.0
79	10	2, 12	2	Whitish ?	2	—A, O C	Green	4.7	3.9	4.3	0.9	1.1	0.0
80	12	4	2	Mucgy ?	2	A, O C	Green	0.2	0.5	3.3	0.2	—0.1	2.0
81	8	2	2	Mucgy ?	2	A	Green	0.3	0.5	0.0	0.0	0.0	0.0
82	12	1	2-5	Usual	2	—A, O C	G → haze	3.3	3.7	5.2	0.1	1.2	0.1
83	200	12, 20	2-5	Usual	2	A/2, C 2	Green	0.1	5.4	3.0	0.1	1.1	0.1
84	100	40, 20	2-5	Usual	2	A/2, C 5	Green	0.0	5.5	3.9	0.1	1.9	0.0
85	80	12, 20	10	Usual	2	A, O C	Green	1.6	4.5	3.1	0.2	—0.5	0.0
86	80	12, 8	10	Usual, flocculent	—1	No change	G → Hemolysis ?	0.2	0.1	—0.1	0.0	2.4	0.2
87	20	12, 20	10	Usual, flocculent	—1	No change	No growth ?	0.0	0.3	0.0	1.2	2.3	0.1
88	100	12, 4	None	Usual	2	A/2, C 5	No color → G ?	0.0	1.0	3.5	0.1	1.1	0.0
89	100	12, 4	5-10	Usual, flocculent	2	A/2, C 5	No color → G ?	0.0	1.3	3.3	0.2	1.1	—0.1
90	102	20	2, 20	Usual	2	—A, O C	No color → G ?	4.5	3.1	3.3	0.5	0.1	—0.1
91	15	5	None	Mucgy	1	—A, C ? 10	—	3.8	1.0	5.1	0.0	3.0	0.2
92	8	15	None	Mucgy	1	—A, C ? 10	—	3.7	0.9	5.0	0.0	3.1	0.2
93	20	4, 12	5	Usual	None	—A/2, C —1	—	4.1	1.5	2.1	3.1	—0.2	1.1
94	10	4, 12	5	Usual	None	—A/2, C —1	—	1.1	1.6	2.7	3.5	—0.1	1.0
95	15	1, 20	5	Usual	None	—A/2, C —1	—	4.9	6.2	3.6	2.3	0.1	1.2
96	12	30	15, 12	None	None	—A/2, C —1	—	1.5	1.8	1.0	1.1	0.0	1.0
97	13	30	12, 1	5	None	—A/2, C —1	—	1.9	4.7	3.3	1.5	0.0	1.0
98	11	20	4, 10	None	None	—A/2, C —1	—	1.5	1.7	3.1	2.1	—0.1	1.1
99	15	30	4, 12	57	Whitish, D	—A/2, C —1	—	5.0	1.0	3.2	3.2	0.0	1.1
100	16	8	2, 1	5	Mucgy	—A, C 5	—	1.5	3.2	1.8	3.7	1.7	3.5
101	115	10	12, 20	None	—1	A, C 3	Green	0.2	1.7	3.5	0.0	1.1	0.0
102	116	60	12, 20	None	—1	A, C 3	Green	0.0	4.9	3.5	0.0	1.4	0.0
103	117	60	6, 12	—1	Usual	—A, O C	Green	3.8	4.6	1.0	0.1	0.1	0.1
104	60	20, 8	None	—1	Usual	A, C 3	Green	0.3	1.7	3.5	0.2	1.5	0.0
105	118	10	20, 12	None	—1	A, C 3	Green	0.0	5.0	3.1	0.1	1.1	0.2
106	1315	8	2	3	Usual	—A, O C	No color	5.2	1.7	—0.1	3.5	0.1	0.0
107	135	60	4, 5	None	Usual	A	No color	0.0	1.0	0.3	0.3	1.5	0.2
108	136	10	2	None	Usual	A, C —1	No color	3.5	3.9	1.1	0.3	3.1	0.0
109	137	20	8, 12	None	Usual	A, C 3	No color	0.0	1.7	3.4	0.1	1.5	0.1
110	138	120	12, 20	None	Usual	A, C 3	† Color	0.0	1.8	3.3	0.2	1.2	0.0
111	139	40	12, 20	None	Usual	A, C 8	† Color	0.0	1.7	3.1	0.3	1.1	0.0
112	140	30	12, 20	None	Usual	A/2, 13	† Color	0.0	4.8	8.1	0.1	1.1	—0.1

STRAINS FROM HUMAN THROAT

STRAINS FROM HUMAN FECES

STRAINS FROM BLOOD, PATHOLOGIC CONDITIONS, ETC.

28	10	4, 6	10	Muggy	—1	A, H ₂ O, C 3	Green	5.2	1.8	5.3	0.7	3.4	0.1
38	10	1.8	5	Muggy, some cone rings	None	—A, 0 C	Hemolysis	3.0	2.7	2.7	0.4	0.2	0.0
10	25	2.8	5	Heavy margin	None	—A, 0 C	Haze -} hemolysis	3.4	2.8	2.8	0.5	0.2	0.3
41	12	1.2	3	Thin, irregular margin	None	A, C 3	Haze -} hemolysis	5.4	4.5	0.1	1.8	0.2	0.2
12	12	1.8	3	Thin, irregular margin	None	A, H ₂ O, 1'	Green, haze	5.1	4.5	0.2	4.6	0.1	0.1
104	40	12, 8	5	Flocculent	5	No change	Brown	3.4	0.2	4.7	0.1	0.2	0.1
105	12	2.1	None	Muggy	1	No change	No color	3.3	3.5	3.9	0.1	3.0	4.8
106	50	8, 12	None	Usual	—	—A, D 1	No color	3.6	3.4	3.8	0.2	1.5	2.7
107	10	4, 8	None	Muggy	2	—A, d C	No color	3.6	3.3	3.7	0.1	2.1	3.3
108	10	—	None	Usual	2	—A, 0 C	No color	3.8	3.3	3.0	0.2	3.1	3.5
109	10	2, 4	None	Usual	2	—A, d C	Green ?	3.5	3.1	3.7	0.2	3.3	1.2
110	10	2, 1	None	Usual	2	—A, 0 C	Green ?	3.6	3.7	3.8	0.2	3.1	3.3
111	12	4, 8	None	Irregular margin	1	—A, d C	Brown ?	1.8	3.1	3.2	0.2	3.1	0.6
112	24	8, 12	None	Usual	2	No change	Green, hemolysis	2.3	0.4	3.1	0.2	2.1	0.6
113	12	2	None	Muggy	2	A, H ₂ O, C 3	? Color	0.1	4.7	4.7	0.5	1.0	0.0
111	12	2, 4	None	Usual	2	No change	Brown	3.6	3.5	3.5	0.2	1.0	1.0
50	8	—	1-2	Usual	2	A/2, C 3	Green	5.7	1.3	5.1	0.2	3.7	0.3
67	8	2, 4	5	Usual, heavy margin	None	—A, 0 C	Green	3.2	3.3	—0.1	3.9	0.1	0.2
58	20	1, 2	5	Usual	—1 liq.	A, H ₂ O, C 2	Hemolysis, whitish	4.9	3.8	5.5	0.7	5.6	0.3
17	20	8, 4	7	Usual -} Hay	1	A, C 18	Green	0.2	3.5	0.0	0.1	0.0	0.2
9	20	5, 4	10	Usual	5	No change	Brown -} haze	2.2	—0.1	1.6	1.7	0.1	0.1
30	24	1, 12	3	Hewley 1	—1	No change	Yellow -} haze	1.5	1.8	1.5	0.3	0.1	0.3
20	20	8, 12	1	Usual	None	—A, d C	Haze	1.3	2.1	2.6	0.3	0.4	0.1
3	8	2, 1	None	—	—	A, D 1	Green	3.7	3.5	4.1	3.1	2.8	2.8
1	8	2, 4	None	—	Whiter	A, C 1	Green	4.3	3.6	4.3	2.6	3.0	2.7
5	III	2, 8	None	—	—	A, C 1	Haze	3.7	3.7	1.9	3.2	3.0	2.2
6	100	10, 20	3	Concentric rings, irregular margin	—	A, C 1	Green	3.5	0.0	0.5	2.9	0.1	0.2
32	40	20, 2	—	Concentric rings	—	A, C 1	No color	0.0	2.6	0.1	0.1	0.1	0.1
30	41	12, 15	None	Centered ?	—	Alk, 0 C	Haze -} hemolysis	4.0	3.0	2.7	0.2	1.1	0.2
31	10	—	None	Centered ?	—	A, C 1	Haze	5.0	4.1	1.0	2.6	0.2	0.5
14	30	2, 1	None ?	Unfavorable	liq.	A 3, H ₂ O base, C 3	? Color	1.9	3.0	1.7	0.4	3.5	0.5
15	40	2, 1	None ?	Unfavorable	liq.	A 3, H ₂ O base, C 3	? Color	1.7	3.0	5.0	0.5	3.7	4.7
17	24	1, 8	None	Usual	—	No change	—	3.1	2.0	3.0	0.0	2.6	3.0
10	25	8, 12	1-2	Usual, irregular margin	None	No change	Hemolysis	2.0	0.1	0.0	0.3	0.0	0.0
20	8	2, 1	None	Usual	2	A, C 2	Green	1.0	3.7	1.3	1.3	2.9	1.8
21	8	2, 1	None	Usual	2	A, C 2	Green	1.3	1.1	1.3	1.1	2.7	1.3

STRAINS OF UNCERTAIN ORIGIN

181	30	2, 12	None	Muggy	3	A, 0 C	G - haz	2.7	2.2	2.2	0.3	4.0	0.1
185	30	2, 12	10	Mugy	3	A, 0 C	G - hemolys	2.4	2.3	1.8	0.1	1.8	0.1
31	8	2, 1	0	Usual	2	A, 0 C 10	Green	5.3	1.4	1.5	0.1	3.5	0.1
33	100	20, 16	10	Mugy	-1	A, 0 C	Green	1.0	1.5	5.1	0.8	3.3	0.1
51	50	8, 12	10	Mugy	6-1	A, 0 C	Green	5.2	6.1	6.7	2.2	0.5	0.5
36	4	2	None	Mugy, yellowish	10-1	A/4, C 10	Green	1.6	1.1	0.2	0.5	0.1	0.1
37	8	2, 6	None	Mugy	1	-	B - haz	1.0	1.2	4.1	0.2	3.1	0.1
103	50	1, 12	27	Thin, irregular margin;	None	A, 0 C	Brownish	3.8	4.1	3.3	3.4	0.0	0.0

from horse feces and from throats (of cats, dogs, and man), rather easily from normal human feces, and with difficulty from other fecal material (of cats, dogs, and especially cows).

Strains from a given region of any one individual are markedly alike, morphologically and physiologically; strains from the various regions of a given individual may all resemble each other very closely,

Sample specimens from a given throat yield strains which usually vary more, physiologically and morphologically, than those from any other single sample.

Human throats and pathologic sources yield approximately 50 percent of the strains clearing in broth in ten days. Cat and dog throats yield 20 to 30 percent; for the other regions of the alimentary canal of animals, the averages are much lower; clearing strains are not uncommon in the feces.

Pathologic samples yield the greatest proportion of hemolyzing strains. They may occur in throat samples, and in those from the alimentary canal. A high percentage of hemolyzing strains was found in dogs—32 percent of 101 strains from the stomachs and intestines of eight dogs. Hemolysis does not seem to be correlated with the results in litmus milk, gelatin, or with any of the Gordon reactions or complexes.*

* This lack of correlation is illustrated by the fact that the seventy-six hemolyzing strains isolated fall into fourteen different fermentative complexes, many of but one or two strains each. The most common complexes for these hemolyzing strains are given as follows:

(a) Saccharose-lactose-salicin for 31 strains (40%) from dog stomachs; 6 strains (7%) from dog throats; and 2 strains (2%) from blood, etc.

(b) Saccharose-lactose-salicin-mannite for 3 strains (3%) from blood, etc.; 3 strains (3%) from human feces; 2 strains (2%) from pigeon intestine; 1 strain (1%) from cat throat, and 1 strain (1%) from dog throat.

(c) Lactose-salicin-mannite for 6 strains (7%) from human feces, and 3 strains (3%) from dog throats.

(d) Saccharose-lactose-salicin-raffinose-mannite-inulin for 2 strains (2%) from dog throats, and 1 strain (1%) from hen intestine.

As to sources: (a) is not what one would expect of *Streptococcus pyogenes*; (b), which has to do with *faecalis*, is more within the expected range of habitats or sources; (c) is *gracilis*, except that it lacks the ability to liquefy gelatin, and its contributing sources support its position as a variety of *faecalis*; (d) is not the fermentative complex usually attributed to hemolyzing strains. The question of relation between hemolysis and pathogenicity is entirely outside my investigations. But the correlation of hemolysis with the saccharose-lactose-salicin complex is so generally accepted (e. g., Lyall finds that 74 percent of his hemolyzing strains fall into this complex) that it seems worth while to point out that less than 50 percent of my hemolyzing strains are limited to the saccharose-lactose-salicin complex. Floyd and Wolbach (1914) state that hemolysis is, in general, a characteristic of pathogenic streptococci, but add that the property of hemolysis is not characteristically associated with the fermentative complexes. Often both hemolytic and pathogenic strains have a wider fermenting range than is generally believed, or than the figures of Lyall, Davis, and Floyd and Wolbach would indicate. Heidelck (1913), for instance, reports for his 21 pathogenic strains fermentation of all six of the Gordon substances discussed in my paper. Six of Holman's eleven strains fermented mannite; over half of Thro's thirty strains fermented raffinose or mannite, or both; Hülpers (1911) reports raffinose and mannite both for streptococci pathogenic to rabbits.

See Lyall for the relative values of blood agar and blood broth. I found on blood agar a distinct type—causing neither hemolysis nor green color, but a very fine delicate growth, seen only on the upper surface. It occurred in long-chained streptococci, and, while definite, the growth was so delicate as to be unobserved in pour plates, tho plainly present in streak plates. These constituted about 11 percent of the strains tested on blood agar; while more common from the mouth than from any other source, they are not limited to that habitat. They cover a wide range in their fermenting powers, and they usually ferment raffinose or mannite.

Litmus milk does not seem to be very closely correlated with the origins of the streptococci or with their fermentative reactions. This is noticeably true with regard to litmus milk and lactose.*

Strains from pathologic conditions and from throats often fail to develop in gelatin; strains from all other sources usually grow readily at room temperature. Only twenty-one of the 554 strains liquefy gelatin†; tho short-chained forms, they have apparently no correlated characters of any value. It is rather strange that among my numerous fecal and intestinal strains there is not one strain conforming to the *gracilis* type. (If the gelatin character is ignored, this type is well represented.)

A comparison of the amounts of acid formed yields little of value. Saprophytic strains (including those from the feces and the alimentary canal) are usually high fermenters (3.0 to 5.0 or even 6.0). But a careful analysis of my strains does not warrant for any habitat comparative quantitative statements similar to that made by Hilliard (1913) when contrasting throat and milk strains: "Throat streptococci, on the other hand, seldom yield over 2.5 percent acid in any substance."

Morphologic characteristics are not, independently or in connection with other characteristics, definitely differential.‡

CLASSIFICATION

The six species recognized by Andrewes and Horder (one of the original seven has been placed under *Pneumococcus*) have been used as a starting point¶ by several later workers. Winslow (1908) recog-

* Floyd and Wolbach state that in certain instances the media showed acid production where no change was produced in the milk, and occasionally acid was produced in milk without the fermentation of lactose in serum water. Shorer (1912) states that the milk acid formed is usually lactic acid, but that coagulation may occur as the result of the formation of amino-acids (and possibly other causes). In my own work, of about 400 strains tabulated in this connection 6 percent failed to ferment lactose and 30 percent to coagulate milk. Half of this 30 percent fell below 2.0 in their lactose titration records, and over two-thirds below 2.6; yet strains very often coagulated milk with lactose-acidity records below 1.9; and at least twenty-seven tubes failed to coagulate milk (in ten days) with high lactose records for acidity (ten tubes ranging from 3.1 to 3.5, nine from 3.6 to 4.5, and eight from 4.6 to 5.5).

† They do not conform however to the Winslow fermentative limitations of *Streptococcus gracilis*, as all of them fermented saccharose. They were distributed as follows: dog throat, 8 strains; dog alimentary canal, 2 strains; pigeon and hen alimentary canals, 7 strains; blood, etc., 3 strains; uncertain origin (stock culture from another laboratory), 1 strain.

‡ In the sources examined, wide, hazy capsules (such as are usually attributed to *Streptococcus mucosus*) were rare, and they were practically limited to throat and to pathologic conditions. Intestinal streptococci were usually larger and shorter-chained than throat forms. The conglomerate character was most common in throat strains. Cell-division at right angles to the chain length was more common in intestinal strains.

¶ It must be admitted that the classification of Andrewes and Horder does not work out very satisfactorily when all of the Gordon test substances are used. The number of variants is too large. In Gordon's paper on the scarlatina strains, for instance, anginosus is represented by 54 strains, 3 conforming to the type and 51 variants; and salivarius by 37 strains, 4 typical strains and 33 variants! With fewer substances, however, the matter is less hopeless. Every method of classification has its troublesome intermediates; and they are as numerous and as troublesome in the biometric method as in any other. In this connection, it will be helpful to recall Bergson's statement, written, of course, in an entirely different connection: "The group must not be defined by the possession of certain characters, but by its tendency to emphasize them."

nized also *gracilis* of Escherich and others. Bergey, Hilliard, and others have modified somewhat the physiologic attributes of one or more of these species. For example, Bergey drops salicin from the *pyogenes* characters, and Hilliard raffinose from those attributed to *anginosus*. Here, as in other fields of biologic nomenclature, it is a question how far subsequent modification of described species may go. I have indicated in Table 16 the larger groups into which my 767 strains fall, using the Winslow classification plus two fermentative combinations common in my own strains. The number of strains from the various habitats or sources varies so greatly that the percentage is given, because the percentages are more nearly comparable than the actual numbers of strains would be.

A glance at this table shows that the main representatives of a species are usually from the habitat designated by Andrewes and Horder; for example, 28 percent of the human throat strains fall under *salivarius*, and the only large group under *equinus* came from equine feces.

On the other hand, strains from a selected habitat are scattered through a number of species; for example, human throat strains are also found under *mitis* (14 percent), still leaving 47 percent unplaced. It will be seen that several other habitats are barely represented in these seven groups; for example, but 22 percent of the bovine fecal strains appear in these seven species.

It will be noticed however that the combinations most frequent here are also those occurring most often in the "family-tree" worked out earlier (Chart 1).

Altho these fermentative complexes are not definitely diagnostic, there are interesting relations suggested. For example, most of the mouth strains, 85 percent, belong to the saccharose-raffinose branch. (Ten percent of the mouth strains fermented none of the six substances. This makes a possible total of 95 percent of the human mouth strains.) And 74 percent of Hilliard's mouth strains belong here also. The other branches are less characteristic. In the lactose-mannite branch we find 53 percent (meat extract) and 86 percent (meat) of the human fecal strains, 64 percent of the blood strains, and 77 percent of the dog throat strains.

The large number of bovine fecal strains which yield the saccharose-lactose-salicin-raffinose combination led Prof. C.-E. A. Winslow to suggest *Streptococcus bovinus* as an appropriate name. This pro-

portion of bovine fecal strains fermenting raffinose is even more marked in the work of other investigators. Another nameless and important combination is the saccharose-lactose-salicin-raffinose-mannite one. Dog throat strains form the most prominent contributions,

TABLE 16
PRINCIPAL SOURCES CONTRIBUTING THE COMMON FERMENTATIVE COMPLEXES OF STREPTOCOCCI

Names of Species of Streptococci	Gordon Substances Fermented	Largest Group Found in My 767 Strains	Group Second in Rank	Group Next in Rank
Equinus	Saccharose, salicin, but not lactose	15% equine feces...	5% human feces...	4% blood, etc.
Mitis	Saccharose, lactose, and salicin	70% feline feces...	42% feline alimentary canal	20% milk; 14% human throat; 12% human feces; 10% canine feces; 8% blood, etc.; 7% feline throat; 6% bovine feces; 3% canine throat
Pyogenes	Saccharose, lactose, and salicin. Hemolysis	31% canine alimentary canal	7% canine throat...	
Salivarius	Saccharose, lactose, and raffinose	28% human throat..	29% feline throat...	9% human feces; 6% blood
Anginosus	Saccharose, lactose, and raffinose. Hemolysis			
Gracilis	Lactose and salicin? and mannite? Gelatin liquefied			
.....	Lactose, salicin, mannite, but gelatin not liquefied	39% canine feces...	24% feline alimentary canal	23% human feces; 12% canine throat; 4% blood
Fæcalis	Saccharose, lactose, salicin and mannite	45% feline throat...	36% human feces...	34% blood, etc.; 31% canine throat; 22% milk; 18% feline feces; 17% canine alimentary canal; 16% bovine feces; 13% equine feces
Versatilis	Saccharose, lactose, salicin, raffinose, and mannite	34% canine throat..	29% equine feces...	16% bovine feces; 14% canine alimentary canal; 14% blood; 11% milk; 5% feline alimentary canal; 5% feline throat
Bovinus	Saccharose, lactose, salicin, and raffinose	46% bovine feces...	16% human throat..	11% equine feces; 5% canine alimentary canal; 4% feline alimentary canal

but, tho eighty strains were studied, they represent only about a dozen dogs (studied at varying intervals). If a name is given to this combination, it should indicate the as yet unnamed fermentative combination, raffinose and mannite. Since we have already several names based

mainly on fermentative activities,* it might not be out of place to suggest also as a name for these strains, representing nearly 10 percent of my 767 strains, *Streptococcus versatilis*. If the fermentative limitations stand, these names will be needed; if they are eventually discarded for agglutinating or other characteristics, the reclassification will be such a wholesale readjustment that one or two names more will be immaterial. To prove or disprove the value of these fermentative limitations of species of streptococci, we must have some way of designating the main groups, and the two names suggested here supply that lack.

Nearly 700 of my 767 strains are included in the seven old species and the two new ones in this paper. About a dozen failed to ferment any of the six Gordon substances. Non-fermenters have been reported by many working with meat extract media. The differences obtained with meat media and meat infusion media might make it questionable whether the Andrewes and Horder and the Winslow classifications can be used for my meat results, even tho the groups are apparently well represented. Floyd and Wolbach give a large number (over 60 percent) which do not ferment the six Gordon substances discussed here and 6 percent that fail to ferment any substance, even dextrose and milk. In view of the fact that they used Hiss serum water, these results are difficult to explain. My own work does not warrant making a new species for these non-fermenters, and therefore it seems wiser to leave this question for the present.

COMPARED GORDON REACTIONS OF VARIOUS INVESTIGATORS

My earlier work with meat extract media and with meat media indicates that it would be almost impossible to compare the work of the various investigators, especially when we have the added difficulty of qualitative vs. quantitative determination of acid. Nevertheless, I have prepared a table listing the various investigators who have dealt with the streptococci and their fermentative reactions, giving the total number of strains each added to this work, and the relative availability of saccharose, lactose, raffinose, and mannite.

The second half of the table includes the fermentative groups most prominently represented in the strains. (Note that inulin has been omitted from the comparisons so as to bring most of the strains upon the same basis; Winslow and

* No attempt is made to pass upon such species as *mucosus* and *viridans*; in the latter, for example, green pigment is associated with so many different fermentative complexes that it cannot be considered in this connection.

TABLE 17
COMPARISON OF FERMENTATIVE COMPLEXES OF VARIOUS INVESTIGATORS *

Source	Worker	Strains	Samples	Gordon Media Foundation	Acid Estimated	Percentage Fermenting			
						Lac.	Sac.	Man.	Raf.
	Andrewes and Horder	94	-	Meat ?.....	Litmus	33	68	8	22
Air.....	Gordon.....	107(200)	Extract.....	Litmus	0?	65	0?	0?
Water.....	Houston.....	45(52)	23	Extract.....	Litmus	Most	Most	Most
Milk.....	Savage.....	21	Meat.....	Litmus	100	60	44	23
	Houston.....	172	100(?)	Extract.....	Litmus	97	90	20	19
	Hilliard, Stowell and Schlesinger.....	55(46)	100	Extract.....	Titration	91	59	0	1
	Rogers and Dahlberg	42	25	Extract (phos.)	Titration	100	50	69	4
	Broadhurst.....	120	112	Meat.....	Titration	78	66	33	31
	Broadhurst.....	13	12	Extract.....	Titration	84	62	53	7
Mouth:									
Human..	Gordon.....	300(?)	22	Extract.....	Litmus	60?	100	0	?
	Bergey.....	63(65)	17	Hiss; pept.....	Litmus	87?	75?	50?
	Hopkins and Lang...	10	10	Meat.....	Litmus	100	100	90
	Broadhurst.....	43	14*	Meat.....	Titration	81	86	0	62
	Hilliard, Stowell and Schlesinger.....	185(163)	?*	Extract.....	Titration	73	69	0	44
	Gordon.....	155	38*	Extract.....	Litmus	100	97	1	27
	Cumpston.....	80	25*	Extract.....	Litmus	100	96	18	10
Bovine...	Rogers and Dahlberg	39(40)	21	Extract (phos.)	Titration	100	89	87	43
Canine...	Broadhurst.....	80	31	Meat.....	Titration	94	80	85	32
Feline....	Broadhurst.....	51	26	Meat.....	Titration	100	97	56	30
Esophagus to large intestine:									
Canine...	Broadhurst.....	101	40	Meat.....	Titration	95	80	53	39
Feline....	Broadhurst.....	85	34	Meat.....	Titration	97	50	32	14
Fowl....	Broadhurst.....	16	8	Meat.....	Titration	87	100	68	50
Fecal:									
Human..	Houston.....	300(229)	15-20	Extract.....	Litmus	100(76)	85	24(29)	32
Human (sewage)	Houston.....	100	100	Extract.....	Litmus	100	45?	4	100
Human..	Winslow and Palmer	116	15	Extract.....	Titration	62	...	28	6
	Fuller and Armstrong	123	?	Extract.....	Titration	94	90	65	0
	Clemesha.....	115	11?	Extract.....	Litmus	92?	92?	92?
	Broadhurst.....	38(39)	24	Extract.....	Titration	100	65	81	2
	Broadhurst.....	31	10	Meat.....	Titration	90	64	40	83
Bovine...	Houston.....	100	10?	Extract.....	Litmus	100	94	0	74(78)
	Winslow and Palmer	86	21	Extract.....	Titration	52	...	6	28
	Fuller and Armstrong	97(98)	?	Extract.....	Titration	77	75	3	73
	Rogers and Dahlberg	114	56	Extract (phos.)	Titration	100	98	18	96
	Broadhurst.....	30	23	Extract.....	Titration	76	76	23	53
	Clemesha.....	39	11	Extract.....	Litmus	99	99	99
Canine...	Broadhurst.....	38	11	Meat.....	Titration	94	55	63	30
Feline....	Broadhurst.....	11	2	Meat.....	Titration	100	90	20	0
Equine...	Winslow and Palmer	100	12	Extract.....	Titration	8	...	2	4
	Bergey.....	11(14?)	Hiss; pept.....	Litmus	100	42?	28?
	Fuller and Armstrong	129	?	Extract.....	Titration	24	46	2	12
	Broadhurst.....	44	35	Extract.....	Titration	61	84	45	47
Blood, pathologic conditions (see mouth above):									
	Andrewes and Horder	228(300)	200?	Extract.....	Litmus	90?	90?	20?	20?
	Rogers and Dahlberg	51	19	Extract (phos.)	Titration	94	78	27	0
	North.....	19	14?	Hiss.....	Litmus	100	100	45
	Floyd and Wolbach..	247	247?	Hiss.....	Neutral red	34	...	0	15
	Hopkins and Lang...	87	87	Meat.....	Litmus	95	98	11	20
	Thro.....	30	Meat (agar)...	Litmus	50	40
	Lyall.....	246	Ser. and pept..	Litmus	15?	22?
	Ruediger.....	124	124?	Extract.....	Litmus	100	100	50	50
	Cumpston.....	20	20?	Extract.....	Litmus	100	100	10	0
	Beattie and Yates....	42	42?	Extract.....	Litmus	92	97	19	37
	Broadhurst.....	52	35	Meat.....	Titration	85	79	38(48)	26
	Heidelck.....	21	21	Litmus	100	100	100	100
	Buerger.....	33	?	Meat; serum....	Litmus	90?	90?	27?
	Davis.....	88	?	Extract.....	Litmus	94	...	12	0

* Several records are omitted or left incomplete; for example, Bergey (92 strains) and Saito (22 strains), Davis (88 strains), and Buerger (33 strains), whose reports lack the necessary details for comparison here. No in Sections 1 to 4, no percentage lower than 3 is given, nor any percentage based on less than three strains; in Gordon and Andrewes and Horder split their fermentative groups with so many variants that their totals, as an overlapping in percentages, etc., so that an accurate division here is not possible.

Hiss means Hiss Serum Media; "phos." means that dibasic phosphates were added.

In some cases, the figures available do not allow separation of the strains according to origin; that is, "normal * Pt. path.

TABLE 17
COMPARISON OF FERMENTATIVE COMPLEXES OF VARIOUS INVESTIGATORS *

Section 1								Section 2					Section 3					Section 4	
0	Lac.	Sac.	Lac. Sal.	Sac. Lac.	Sac. Lac. Sal.	Sac. Sal.	Sal.	Lac. Raf.	Lac. Sal. Raf.	Sac. Lac. Raf.	Sac. Lac. Sal. Raf.	Sac. Sal. Raf.	Lac. Man.	Lac. Sal. Man.	Sac. Lac. Man.	Sac. Lac. Sal. Man.	Sac. Lac. Raf. Man.	Sac. Lac. Sal. Raf. Man.	
20+	..	3	..	4	9	20	12	6	9	6	7			
..	..	8?	57?	34?										
..	[†]									
..	28	..	23	14	..	23	
..	12	29	5	7			
7	24	42	10	3									
..	33	16	..	40	4	
6	6	..	8	4	20	..	9	3	19	4	
15	22	53	11	
..	27	14	5								
..	26?	12?	..	12?	11?	22?								
..	30	30	13?(24)	30								
9	11	14	30	16								
17	9	16	7	12	30								
..	28	41	9	16								
..	6	61	5	16	..	10	22	
..	10	5	42	34	
..	10	3	..	12	..	31		
..	7	29	3	..	45		
..	10	..	33	5	17	..	14		
..	22	..	42	4	24	5		
..	25				43				
..	17	..	26	21	8	..	24			
..	2	49	..	44	..	23			
22	33	5			
[4]	92	[85]	..	[3]		
..		
..	10	..	7	21	..	55	..		
..	12	..	16	9	19	25	..	16	..		
45	26	13	75	3		
9	[21]	..	3	5	
..	10	4	3	..	69	3	..	10	3	
..	70	6	16	
..	6	46	16	..		
..	99		
..	10	25	39	..	16	..		
88	5	70	18		
..		
9	..	10	56?	26	7	7		
6	10	31	4	8	4	13	..	29		
..	15	6	11				
..	19(46)	3(45)	2(8)	..		
..	15	52	21		
61	12	..	2	21	31	26	21		
..	11	58	11	3	9	5	5	..	3	
[6]	[10]	8	..		
[17]	[15]	[26]		
..	50	50	..	7		
..	70	20	10		
4	12	14	28	14	19	11	..	4		
..	6	8	4	6	4	..	34	..	14		
..	100		

who omitted either mannite or raffinose; and Salomon, who used 10 percent solutions of the test substances; also habitat groups of less than ten strains (e. g., North, White and Avery, 9 milk strains) are included in this table; small or very varied lots, therefore, the percentages do not total 100.

given here, do not always approach 100 percent. These workers have also later regrouped their results, with often

and infected throats," Hilliard, "scarlatinal throats and lesions," Ruediger.

Palmer used only lactose, raffinose, and mannite; Thro and Lyall used salicin, raffinose, and mannite only).

This last part is arranged in four longitudinal divisions or sections: (1) including those strains fermenting none of the five substances or only the disaccharids (saccharose and lactose) or salicin; (2) those fermenting raffinose and one or more of the three substances in the preceding group; (3) those fermenting mannite and not raffinose, but fermenting one or more of the substances in Group 1; and (4) those fermenting both mannite and raffinose with one or more of the Group 1 substances. The strains in brackets belong in that main longitudinal division or section in which they occur, but of which subdivision is uncertain because of the smaller number of media used.

This totals over 5,200 strains. If strains isolated, cultivated, and tested in such different ways (as those already noted in the first part of my paper) can be compared, this array of strains ought to be helpful.

CONCLUSIONS

The most striking observation is that by all methods human throat strains practically fail to ferment mannite. Note, in contrast to this, the large number of mannite fermenters in human feces.

Raffinose fermenters are more characteristic of human throats than of the throats (and alimentary canals) of cats, cows, and dogs. Raffinose fermenters are more prominent in bovine feces than in the feces of other animals. They are strikingly lacking in milk. Mannite fermenters are most often found in bovine mouths, human feces, and in milk. Mannite and raffinose have been emphasized by most workers as the rocks of differentiation. Milk strains are conspicuously not raffinose fermenters and rather commonly mannite fermenters. Note here that a large number of fecal strains fail to ferment raffinose or mannite.

It is noticeable that the throat and fecal strains from the same animal species may differ markedly; for example, compare human throat and fecal strains with regard to mannite, or bovine throat and fecal strains with regard to raffinose.

Many of the strains from pathologic conditions fail to ferment raffinose and mannite. But the saccharose-lactose-salicin combination is as commonly the limit of throat strains as of pathologic strains; and 40 to 70 percent of various intestinal and fecal strains fall within this limit, too. This is not in accord with current statements.

The non-lactose character of equine fecal strains seems to have been founded upon an insufficient number of strains (Andrewes and Horder, thirteen colonies; number of contributing samples not given). My percent of non-lactose strains is much lower; it was obtained with meat extract Gordon media. These streptococci are

often very large and in very long chains—larger and ranker than most streptococci, apparently. The large percentage of non-fermenters in the equine strains of Winslow and Palmer is doubtless due to the fact that they did not use salicin and saccharose; in that case their present non-fermenters would be found 1 to 7 columns to the right.

There are many striking (and erratic?) differences, due probably to differences in media and indicator chiefly. The large number of non-fermenters of Floyd and Wolbach is not easy to explain, since it evidently represents almost as many samples as strains. (As noted elsewhere, it may be due to the indicator.) Heidelck finds that all his pathologic streptococci ferment both raffinose and mannite. Clemesha's figures seem too concentrated. He used few strains, from few samples. Fuller and Armstrong do not indicate the number of samples contributing to their strains.

These fermentative tests do not seem to be definitely helpful in indicating the origin of a given streptococcus; to illustrate, mannite streptococci in milk may imply either a bovine buccal origin or human fecal one. A raffinose strain (rare in milk) may indicate human or bovine fecal pollution. It would seem therefore impossible to determine positively the real origin of a strain isolated from milk, water, etc. Interesting as these comparative physiologic results may be, they are not adapted for direct sanitary application.

APPENDIX

DETAILS OF TECHNIC NOT INCLUDED IN THE TEXT

Acidity Division Line.—While 1.2 and 1.5 determined biometrically were used in my earlier papers as the division line between fermenters and non-fermenters, a careful analysis of 4,000 of the tubes selected from the low fermenters shows that arbitrarily adopting the accepted neutral point for litmus, 0.8, as a standard does not shift more than two dozen strains out of the whole lot.

Capsules.—(A) For abdominal insertion, these were made (1) by tying on a small glass tube one drum end of parchment (cultures grown in these tubes were later sealed in with celloidin); (2) by the usual method of forming capsules, namely, rinsing the inside of a clean tube with celloidin, tying top, and then sealing as above. This was unsatisfactory; they were evidently too thin, for invading organisms were found.

(B) For feeding by mouth and for the extract experiments, the capsules were made as described by Brown (1914). The capsules were suspended by a string in tubes of dextrose broth, and sterilized. Inoculations were made into the capsule with a stab needle. After incubation for twenty-four hours, these capsules were examined microscopically and sealed with celloidin while still protected by the test tube. Saliva, intestinal extract, etc., were then added to the dextrose surrounding the capsule and the tube was incubated for one to two days. In the feeding experiments, the sealed capsules were wrapped in

fresh meat, and fed by hand to the dog. Later, long capsules (3-4 inches) were made over gelatin-covered rods; these were left unsealed, the base touching the saliva, etc. They were not inoculated until the outer surface of the capsule had dried. Smears for purity were satisfactory in all but one case.

After recovery, the surface of the capsules was first air dried. In the beginning of this work, they were opened with sterile scissors, but later a hot iron rod was used to burn an opening in the top, while the capsule was held horizontally in the forceps. Loops for smears and streaks were taken through this opening at once. The contents of the capsules were then titrated with controls to confirm the supposed exchanges through the capsule.

On opening the capsules, streaks were made at once on plain agar plates. In all cases (by the Brown method and by my open tube modification of it), the plates appeared to be pure cultures. Several fishings made from each plate confirmed this.

Care of Animals Used for Feeding Experiments.—The dogs were kept in sunny runs, of wire, covered with mosquito netting. They were fed and watered from the outside. The runs were entered to clean them, lysol being sprayed over the ground, sides, etc., freely at such times. Milk, water, and oat meal were heated to 90 C., for two minutes, or longer.

The kittens were kept in netting-covered cages, in an immense room. The dishes, milk, and soil pans were all made streptococci-free or else sterilized. The hands, aprons, etc., of the attendant were washed in carbolic acid. No windows were opened as the experiment covered less than two weeks.

Intestinal Extract.—This was made by Mendel's method (grinding entire gut in sand, adding sodium fluorid, etc.) and tested for lactose by Barfoed's reagent to make sure that live enzymes were present. All extracts were prepared in sterile vessels, etc., and examined for streptococci.

Isolation.—Plain meat agar and plain meat broth were used for isolation throughout, except in the 113 strains mentioned. Occasionally, dextrose broth was used also, as a control condition. No selective media were used. The samples were transferred to broth, incubated eighteen to twenty-four hours, examined microscopically, and the likely tubes streaked on plain agar. In the unfavorable tubes, the examinations were repeated in two days and if necessary, in three days. After three days, isolation in such cases was usually very difficult. The streak plates were found to be much more satisfactory than the pour plates. Small, semi-transparent colonies were fished to plain broth, incubated for eighteen to twenty-four hours, and examined microscopically. Then, if apparently pure, second streak plates were made, and a second set (1-3 tubes) of fishings made. This was to insure purity. These second or subfishings were examined, transferred to slant agar, and incubated for eighteen to twenty-four hours, and then used to inoculate the Gordon media, etc.

No organisms were used which did not show chains containing at least six organisms. Four is too low, I think. Capsule stains were used for the last 550 strains. Tho a large percentage of my organisms fermented inulin, the chain length, shape, and non-encapsulated condition of most of the organisms would seem to indicate that I was dealing with streptococci.

Litmus Milk.—To milk 1.5 acid, a 5 percent litmus solution was added until a pastel pink-purple color was obtained. This was sterilized in the way described for the Gordon media.

Media.—Broth from meat infusion was used, except in the 113 strains where meat extract was used. Broth of 1.0 and later of 0.5 was used for isolation. for

determining broth characteristics, and for making the agar (1.5 percent) and gelatin (15 percent.). The agar and gelatin were not corrected for acidity. Both were cleared with egg-white. Slants were not used if the water of condensation had disappeared from the tubes.

Special Media.—Blood agar: horseblood, about 1 c.c. to each plate. Calcium broth: small pieces of white marble were added to plain broth. Gordon media: sugar-free broth plus 1 percent of the test substances. These were sterilized one-half hour on two successive days, and incubated for one day before using. (Less than ten tubes of over 12,000 thus made had to be discarded.) Plain meat broth has been used by some for making the Gordon media, subtracting acids in inoculated control tubes. The effect of acids on the cleavage of some of the carbohydrates would indicate that this is not a wise procedure here.

Sterilization.—All media, except for the first 213 strains, were sterilized in the streaming steam, not in the autoclave. All media, except milk, gelatin, and the Gordon media, were sterilized for one hour on two successive days.

Stock Cultures.—These were kept on plain agar, in closed tin pails, at room temperature. Transfers were made every ten days, incubated for one day, and stored the intervening nine.

Titration.—In the cold, as described in my (1912) paper.

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A BACTERIOLOGIC STUDY OF SECONDARY INVADERS IN HOG-CHOLERA *

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Study of hog-cholera has shown that the disease is complicated by secondary invaders such as *Bacillus suispestifer*, *B. suis*septicus, *B. typhi suis* and *B. enteritidis*. The first organism has been noted so commonly in animals suffering with hog-cholera that some of the early workers attributed the disease to this bacillus. It was not until the work of De Schweinitz, Dorset, and McBryde¹ appeared that the etiology was cleared up. These investigations, followed by those of Hutyra,² Uhlenhuth, Haendel, and others,³ established the feature of secondary invasion.

As yet, attempts to differentiate the organisms found in hog-cholera have not met with success. Apart from *B. suis*septicus, which is placed in the hemorrhagic septicemia group, none of the others can be separated sharply from *B. paratyphosus* B and from bacilli associated with meat poisoning. The morphologic, cultural, and serologic behavior of *B. suispestifer* places it in such close relation to these that a definite separation is not possible with the differential-diagnostic methods now available. The significance of this organism in hog-cholera has long been recognized. It is possible by means of *B. suispestifer* to produce an experimental infection giving rise to pathologic changes which apparently correspond to natural hog-cholera.

The bacillus of hog-cholera (*B. suispestifer*), altho present in a number of cases, is not found invariably in the organs of animals with the disease. In several outbreaks of hog-cholera studied by Smith in America, no such organisms were isolated. Preiss, in a systematic examination of 80 cases from different sources, found *B. suispestifer*

* Received for publication May 28, 1915.

1. Circular 41, U. S. Dept. of Agriculture, Bureau of Animal Industry, Washington, D. C., 1903.

2. Handb. d. Serumtherap., p. 42; Specielle Path. u. Therap. d. Haustiere, 1910, 1, p. 142.

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TABLE 1
POSTMORTEM FINDINGS IN VIRUS HOGS

Hog	Lung	Kidney	Lymph Nodes	Intestine (ileocecal)	Organisms Isolated		
					Lung	Spleen	Intestine
1	++	+	Hemorrhagic	Inflamed	P	P.C	C
2	++	++	Hemorrhagic	Inflamed	P.C	P.C	Negative
3	+++	++	Hemorrhagic	Inflamed	P	P	C
4	+++	++	Hemorrhagic	Inflamed	C	P	Negative
5	++	+	Hemorrhagic	Inflamed	P	P	Negative
6	++	+	Hemorrhagic	Inflamed	Negative	Negative	C
7	+	++	Hemorrhagic	Inflamed	P	P	C
8	++	++	Hemorrhagic +	Inflamed	P.C	P	Negative
9	+	++	Hemorrhagic	Inflamed	P	P.C	C
10	+	++	Hemorrhagic (slight)	Inflamed	Negative	P	Negative
11	+	+	Hemorrhagic	Inflamed	C	Negative	C
12	+	+++	Hemorrhagic	Inflamed	C	C	C
13	+	++	Hemorrhagic	Inflamed	Negative	C	Negative
14	+	+	Hemorrhagic	Inflamed	P.C	C	S
15	+	+	Hemorrhagic	Inflamed (slight)	C	C	C
16	+	+	Hemorrhagic	Inflamed	P	P	P.S
17	+	++	Hemorrhagic	Inflamed	C	C	Negative
18	+	+	Hemorrhagic	Inflamed	C	P.C	C
X	+	+++	Hemorrhagic	Inflamed	Negative	Negative	Negative
19	+++	++	Hemorrhagic	Inflamed	P.C	P.C	C
20	+	+	Hemorrhagic	Inflamed	Negative	C	C
21	+	+	Hemorrhagic	Inflamed	Negative	Negative	C.S
22	+++	+	Hemorrhagic	Inflamed	Negative	S	C
23	++	+	Hemorrhagic	Inflamed	Negative	Negative	C
24	+	+	Hemorrhagic	Inflamed	Negative	Negative	S.C
25	+	+	Hemorrhagic (slight)	Inflamed	Negative	S.C	C
26	+	++	Hemorrhagic	Inflamed	C	C	C
27	+	+	Hemorrhagic (slight)	+	(Petechia)	P	C
28	+	+	Hemorrhagic	+	(Petechia)	P.C	P.C
29	+++	++	Hemorrhagic (slight)	+	(Petechia)	P	P.C
30	+++	+	Hemorrhagic	+	(Petechia)	Negative	C
31	+	+	Hemorrhagic	Inflamed	C	C	Negative
32	+	+	Hemorrhagic (slight)	+	(Petechia)	P.C	Negative
33	+++	+	Hemorrhagic	Inflamed	P	P.C	C
34	+++	+	Hemorrhagic	Inflamed (slight)	P	P	Negative
35	++	+	Hemorrhagic	Inflamed	P	P.C	S.C
36	+	+	Hemorrhagic	Inflamed	P	P.C	Negative
37	++	+++	Hemorrhagic	Inflamed	Negative	P	Negative
38	+	+	Hemorrhagic	Inflamed	Negative	P	C
39	+	+	Hemorrhagic	Inflamed	P.C	P	Negative
40	+	+++	Hemorrhagic	Inflamed	P	P	C
41	+	+	Hemorrhagic	Inflamed	S.C	Negative	C
42	+	+	Hemorrhagic	Inflamed	P	P.C	Negative
43	+++	+	Hemorrhagic	Inflamed	P	P	P
44	+	+	Hemorrhagic	Inflamed	P	P	S.C
45	+	+	Hemorrhagic	Inflamed	P	P	C
46	++	++	Hemorrhagic	Inflamed (slight)	P	C	C
47	+	+	Hemorrhagic	Inflamed	Negative	Negative	C
48	++	+	Hemorrhagic (slight)	Inflamed	Negative	Negative	C
49	++	+	Hemorrhagic	Inflamed	Negative	C	C
50	+	+	Hemorrhagic	Inflamed	C	C	C
51	++	+++	Hemorrhagic (slight)	Inflamed (slight)	P	P	C
52	+++	++	Hemorrhagic	+	(Petechia)	P	C
				and Ulcers			
53	+	+	Hemorrhagic	Inflamed	Negative	Negative	C
54	+	+++	Hemorrhagic	Inflamed	C	Negative	C
55	+++	++	Hemorrhagic	Inflamed (Ulcerations)	P	P	P

+ = petechiae present. ++ = well-developed. +++ = marked. ++++ = extreme.
P = paratyphoid group. C = colon group. S = B. suis septicus.

31 times. Uhlenhuth was able to isolate the organism in 76 of 178 cases. Extraordinary numbers of such bacilli are found in pure culture in the organs of hogs artificially infected with the filtered virus of hog-cholera. From these findings, we are led to infer that normal animals harbor this bacillus; yet, of several hundred normal hogs studied by Uhlenhuth, Hübner, and others, only 8.4 percent showed what appeared to be true *B. suispestifer*. These figures are based on a study of the intestinal contents of the animals.

In the investigation here reported, an attempt was made to classify the chief groups of organisms found in a number of hogs artificially infected with the hog-cholera virus. The lungs, spleen, and intestine of each animal were studied in an effort to correlate the bacterial findings, with the organic lesions. The hogs were so-called virus hogs, inoculated with virus for the production of serum. The animals were killed usually on the eighth or tenth day after inoculation, when the previously observed maximal temperature was on the decline.

TECHNIC

Immediately after bleeding, the organs of each animal were excised and placed in separate containers. With a heated scalpel a small area of the lung and spleen was scorched, the organ broken at this point, and a sterilized platinum needle inserted into the tissue. Smears were then made on Conradi-Drigalski agar plates. In isolating organisms from the intestine, care was taken to avoid fecal contamination. An area was scorched with a heated scalpel and an incision made into the mucosa. With a sterile platinum spatula, the mucous membrane was separated from the serosa and a platinum needle inserted between. Material thus obtained was streaked on Conradi-Drigalski plates. After forty-eight hours' incubation, two colonies were fished from each plate and inoculated on agar slants. Further purification was effected by streaking new plates from each of these cultures, and finally transplanting to agar slants from the colonies developed. These agar cultures were used in the inoculation of the series of differential media.

The media used were Loeffler's malachite green dextrose lactose nutrose solution (Loeffler 1); Loeffler's malachite green lactose nutrose solution (Loeffler 2); Barsiekow's dextrose lactose nutrose litmus solution (Barsiekow 1); Barsiekow's lactose nutrose litmus solution (Barsiekow 2); Hetsch's litmus nutrose mannite solution; Petruschky's litmus whey (Lacmusmolke); and milk, peptone, dextrose, lactose, orcein agar, and neutral red agar.

The inoculated series were incubated at 37.5 C. for one week; then indol tests were made and the other cultural characters noted.

If the data for two of the organs examined are tabulated according to the severity of the lesions, we note the following percentage of cases in which the different bacterial groups were found:

TABLE 2
PERCENTAGE OF CASES IN WHICH THE DIFFERENT BACTERIAL GROUPS WERE FOUND IN LUNGS
AND INTESTINE

Bacterial Groups	Lesions in Lungs					Lesions in Intestine				
	Few Pe- techiae (28)	Well-de- veloped Pe- techiae (17)	Marked (7)	Ex- treme (3)	Total	Slight In- flam- mation (4)	Mod- erate In- flam- mation (44)	Pe- techiae (5)	Pe- techiae and Ulcers (2)	Total
Negative.....	9(32.1%)	6(35.3%)	1(14.3%)	0	16	1(25.0%)	12(27.2%)	1(20.0%)	0	14
B. paraty- phosus.....	9(32.1%)	10(58.8%)	5(71.4%)	3(100%)	27	0	2 (4.5%)	2(40.0%)	1(50.0%)	5
B. coli.....	12(42.8%)	4(23.5%)	2(28.5%)	0	18	3(75.0%)	29(65.5%)	4(80.0%)	1(50.0%)	37
B. suisep- ticus	0	6(13.6%)	0	0	6

The so-called secondary invaders do not seem at first glance to play an important part in inducing tissue changes, if we may judge from the findings in organs which show lesions of varying degree. The lungs, for example, in sixteen cases were sterile; yet they showed petechial hemorrhages and inflammation. On the other hand, the paratyphoid group appears to modify the course of the infection. Table 2 shows how these bacteria, when present in the lungs, were

TABLE 3
LOCALIZATION OF BACTERIAL GROUPS IN DIFFERENT ORGANS

Organ	B. coli	B. para- typhosus	B. suisep- ticus	B. coli and B. para- typhosus	B. para- typhosus and B. suisep- ticus	B. coli and B. suisep- ticus	Negative
Lung.....	11	17	..	10	..	1	16
Spleen.....	11	20	1	12	..	1	10
Intestine.....	30	3	1	1	2	3	14

correlated with a progressive intensity of the lesions. Other factors are present, however, and cannot be eliminated. The colon bacilli are found rather frequently (see Table 3). In the intestine, these organisms may play a more important part since they are naturally associated with the tract. It is possible that they are drawn into the lungs during the bleeding as a result of forced respiration and the flow of blood. The simplest explanation of the presence of these bacteria in the organs is that of metastasis by way of the circulation. The organ-

isms can enter the blood stream readily through ruptured capillaries in the intestine, for example, and so become localized in the different organs.

The frequency with which different groups of organisms were associated with the organs is shown in Table 3. The bacillus coli and bacillus paratyphosus groups were evenly distributed in the lungs and spleen, occurring each alone and in combination. In the intestine, *B. coli* was found most frequently (30 times) and *B. paratyphosus* in but three instances. *B. suis* was isolated in nine cases of a total of fifty-five, the intestine harboring this organism in six cases.

The presence of *B. suis* in hog-cholera does not point, according to some investigators, to an infection with what is known as true swine plague. Uhlenhuth and his co-workers³ state that organisms resembling and behaving like *B. suis* are found normally in the upper respiratory passages and secondarily in the lungs. These bacilli may localize themselves in other organs.

This study does not point to *B. suis* as a very frequent inhabitant in either normal or diseased hogs. Tables 1 and 2 show that there was apparently no causal connection between the presence of this organism and the severity of the lesions. The intestines of the animals from which *B. suis* was isolated showed inflammation and were free from petechiae. No such strains were isolated from intestines having petechiae or ulcers or both.

In an investigation of this nature, we are confronted with the problem of correlating the bacterial findings with our knowledge of what constitutes true hog-cholera. If we accept the classification made by Schern and Stange,⁴ this becomes simpler. They divide hog-cholera into different groups as follows: virus cholera or "viruspest," a disease caused by virus alone; paracholera or "parapest," caused by *B. suis* and other organisms; and hog-cholera or "pest," caused by virus, *B. suis*, and other organisms. Bearing this classification in mind, we can more readily correlate the presence or absence of certain bacteria from organs with extensive lesions or none at all, since it is hardly to be expected that there should be no overlapping of these groups.

CHARACTERISTICS OF ISOLATED STRAINS

In addition to the cultural tests made of these organisms, motility and reaction to Gram's stain were studied. All but ten strains were

4. Ztschr. f. Infektionskrankh. d. Haustiere, 1914, 15, p. 1.

found to be motile bacilli. Of these latter, nine were ovoid, bipolar, gram-negative, non-motile organisms, and one was a gram-negative coccus, coagulating milk. This last-named organism was isolated from the intestine of one of the animals.

SUMMARY BY GROUPS		
<i>B. coli</i>	<i>B. paratyphosus</i>	<i>B. suis</i> septicus
132	106	9

The paratyphoid strains may be correlated according to their action on Hetsch's mannite nutrose litmus solution and their indol production in peptone water, as follows: Of 106 strains, 88 (83 percent) were indol-negative, producing slight gas; 4 (3.8 percent) were indol-negative, producing abundant gas; 9 (8.5 percent) were indol-positive, producing slight gas; and 5 (4.7 percent) were indol-positive, producing abundant gas.

Thus it is seen that the organisms of this group were chiefly slight gas formers which did not produce indol. They were found principally in the lungs and spleen. Those which formed gas abundantly were divided evenly between indol-positive and indol-negative groups. The few organisms which produced indol from peptone with slight gas formation in mannite were members of the paratyphoid B group. Indol production is not generally ascribed to paratyphoid organisms, altho positive findings have been noted in some instances. Poppe,⁵ using different kinds of peptone, found that some paratyphoid varieties were not indol-positive. Ordinary peptone water showed a positive reaction after four to five days. Andrejew,⁶ working with a series of paratyphoid B strains, repeatedly obtained positive indol reactions.

B. paratyphosus A, and *B. paratyphosus* B can be distinguished by their behavior in mannite nutrose litmus solution and Petruschky's litmus whey. The A type does not ferment mannite with production of gas, but gives rise to permanent acidity in litmus whey. The B type produces both acid and gas in the Hetsch medium, and gives to litmus whey first an acid, and later a strongly alkaline reaction, leaving the media deep blue. Within two weeks or so a change to acidity takes place again. Some paratyphoid A strains can produce a small amount of gas in mannite. The few here isolated were doubtless of this type.

5. Ztschr. f. Infektionskrankh. d. Haustiere, 1908, 5, p. 42.

6. Arb. a. d. k. Gsndhtsamte., 1910, 33, p. 1030.

PARATYPHOID STRAINS FORMING INDOL AND THEIR REACTION IN LITMUS WHEY

Lung		Spleen		Intestine	
Acid	Alkaline	Acid	Alkaline	Acid	Alkaline
3	0	3	5	2	2

The indol-forming paratyphoid strains were about evenly divided with regard to their reaction in litmus whey. The spleen was found to contain the true paratyphoid B type more frequently than either the lungs or intestine. Acid formation in litmus whey with the production of indol from peptone may be looked upon as a variation. After four weeks incubation in litmus whey, these cultures were still sharply acid. Any change in the reaction of this very sensitive indicator has been noted invariably within one week. Any possibility that these strains were *B. suipestifer* is eliminated because of their indol-producing character, notwithstanding the fact that the acid reaction was conformable to the behavior of this organism.

The paratyphoid strains may be grouped from the standpoint of reaction to litmus whey and gas production in Hetsch's mannite solution, as follows: Of 106 strains, 90 were whey-alkaline, producing slight gas in mannite; 10 were whey-acid, producing slight gas in mannite; 5 were whey-alkaline, producing abundant gas; and 1 was whey-acid, producing abundant gas. This grouping tends to throw the greatest number of the organisms into a class of gas formers which give an alkaline reaction in Petruschky's medium. Practically all these produced but a slight amount of gas. A few gave an acid reaction in whey and produced little or no gas in the mannite medium. One of these formed no indol and was culturally like *B. paratyphosus* A.

CLASSIFICATION OF ISOLATED STRAINS

COLON GROUP

(1) *Typical B. coli*.—Red colonies on Conradi-Drigalski agar; gas in dextrose and lactose; gelatin not liquefied; indol-positive, non-spore-forming, motile.

Variant A.—Blue colonies on Conradi-Drigalski agar; same cultural characteristics as type; milk not coagulated. Isolated from spleen (one strain).

PARATYPHOID GROUP

(1) *Typical B. paratyphosus*.—Blue colonies on Conradi-Drigalski agar; gas in dextrose; no gas in lactose; non-spore-forming, motile. The "A" type does not form gas in mannite, does not produce indol. The "B" type forms gas in mannite and may produce indol from peptone water.

STRAINS ISOLATED FROM VIRUS HOGS

From spleen (one strain): Acid but no gas in mannite; litmus whey, acid; indol-negative.

From intestine (one strain): Acid but no gas in mannite; litmus whey, acid; indol-positive.

From lung (45), spleen (38), intestine (2): Acid and gas in mannite; litmus whey, alkaline; indol-negative.

From spleen (5), intestine (2): Acid and gas in mannite; litmus whey, alkaline; indol-positive.

From lung (3), spleen (3), intestine (1): Acid and gas in mannite; litmus whey, acid; indol-positive.

From lung (2), spleen (2), intestine (1): Acid and gas in mannite; litmus whey, acid; indol-negative.

SUMMARY AND CONCLUSIONS

Organisms belonging to the bacillus paratyphosus group were chiefly associated with the lungs and spleen of hogs infected with the virus of hog-cholera.

Bacillus coli was frequently found in the lungs and spleen, either alone or in combination with organisms of the paratyphoid group.

Bacillus suisepicus was isolated in few cases—but 9 out of 55—and was found chiefly in the intestine.

Classification of the organisms shows that the greatest number belonged to the paratyphosus B group. The majority of these did not form indol and were found chiefly in the lungs and spleen.

Bacterial findings did not appear to be correlated with the lesions observed in different organs.

The significance of secondary invaders in hog-cholera is not apparent from a study of the lesions and the different groups of organisms isolated.

SEPARATION OF THE ANTIBODY FRACTIONS IN HOG-CHOLERA SERUM *

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The study here reported was undertaken with a view to establishing the protective protein constituents in hog-cholera serum. It was not planned to work out the feature of concentration for practical therapy in hog-cholera at this time, much as it is desired. With the results given here, it is hoped that work will be done along the lines so well laid down by Gibson¹ and Banzhaf,² of the Department of Health of New York City, on the concentration of diphtheria antitoxin. They have demonstrated that artificial concentration is practicable. The procedure is based upon a fractionation of the constituent protein in order to determine the antibody fraction.

The literature on fractional precipitation of antibodies is not extensive. Pick³ finds that diphtheria antitoxin is precipitated from goat's serum in the globulin fraction. Landsteiner,⁴ studying the antitryptic action of blood serum, associates this property with the albumin fraction obtained after removal of the globulins by half saturation with ammonium sulphate. Opsonins in blood serum are said to be linked with the proteins separated by dialysis, as shown by Simon, Lamar, and Bispham.⁵ They report that the euglobulin contains the opsonin, that the pseudoglobulin which remains in solution has none, and that the albumin is inactive. In a study on agglutinins by Gibson and Collins,⁶ it is stated that the relative proportions of the agglutinins in polyagglutinative sera remain constant with regard to the proportional distribution of all of the agglutinins of the serum in the euglobulins and pseudoglobulins. They conclude from these results that it is not trustworthy to differentiate the antibodies contained in the euglobulin and pseudoglobulin fractions. A somewhat later work by Banzhaf and Gibson⁷ gives results which show conclusively that the saturated sodium chlorid soluble serum globulins of the higher fractions are more potent, per gram of protein, in antitoxin than those precipitated by lower concentrations of ammonium sulphate.

The concentration limits at which the several protein fractions will separate by addition of ammonium sulphate differ for various authors. Oppenheimer⁸

* Received for publication May 28, 1915.

1. Jour. Biol. Chem., 1905-1906, 1, p. 161.

2. Ibid., 1907, 3, p. 253.

3. Hofmeister's Beiträge, 1901, 1, p. 351.

4. Centralbl. f. Bakteriöl., I, O., 1900, 27, p. 357.

5. Jour. of Exper. Med., 1906, 8, p. 651.

6. Jour. Biol. Chem., 1907, 3, p. 233.

7. Ibid., p. 253.

8. Grundzüge der Biochemie, 1909, p. 174.

places the limits of precipitation at 25 percent saturation for fibrin-globulin, 25-32 percent for euglobulin, and 32-48 percent for pseudoglobulin. According to Hofmeister and Pick,⁹ one portion of the globulin, the eu-fraction, insoluble in water, corresponds to a precipitate obtained by 28-36 percent saturation with a saturated solution of ammonium sulphate; another part, insoluble in water, the pseudoglobulin, is precipitated by 36-44 percent saturation. Again, we find that seroglobulins can be separated by ammonium sulphate into three fractions, the limits of which are 28-36 percent., 33-42 percent, and 40-46 percent, all of them containing globulin insoluble in water (Porges and Spiro¹⁰). Haslam¹¹ splits serum protein into three bodies by means of ammonium sulphate. Globulin, water-insoluble, will come down with one-third saturation, pseudoglobulin with one-half saturation, and albumin will be soluble in a half-saturated solution of the salt. It is evident that differentiation on the basis of water solubility is untenable for globulin fractions. Results are at variance because of the readiness with which a soluble part becomes insoluble and vice versa. As noted in this study, phenol has a marked effect upon the ease with which the protein goes into solution. Particularly with albumin, ordinarily soluble in water, has this difficulty been encountered. It has been shown by Hammarsten¹² that exposure to the air and holding under water will affect solubilities of globulins. In general, it may be stated that the character of globulin is such that acid will favor its precipitation and lower the precipitation limits, as has been noted by Cohnheim.¹³

The separation of serum proteins is linked with great difficulties. There is always a tendency on the part of a residue of one fraction to remain dissolved in the other. Haslam¹⁴ is of the opinion that proteins exhibit strong affinity for each other, and that a series of precipitations is necessary before a relatively purified fraction may be obtained. In an earlier work, this investigator pointed out that the solubility of globulins is largely increased in the presence of serum albumin and other serum constituents, so that, in an albumin filtrate, considerable globulin may be present. By means of a series of precipitations along fractional lines, he has been able to demonstrate the presence of a protein precipitate in a filtrate supposedly free from other fractions.

EXPERIMENTAL

The chief purpose of this study does not warrant the use of an extremely delicate method of separation of protein fractions since the extent of "contamination" would not affect the specific reaction of the animals to the possible protective property of the fraction. An attempt was made however to arrive at "pure" fractions in so far as this was

9. Hofmeister's *Beiträge*, 1901, 1, p. 351.

10. Cited by Hammarsten, *Textbook of Physiological Chemistry*, 1912, p. 251.

11. *Proc. Physiol. Soc., Jour. of Physiol.*, 1912, 44, p. 241.

12. *Ergebn. d. Physiol.*, 1902, 1, p. 330.

13. *Chemie der Eiweisskörper*, 1911, p. 180.

14. *Jour. of Physiol.*, 1905, 32, p. 268.

possible. The results tend to show that it is possible to obtain pseudoglobulin practically free from euglobulin. This conclusion is based on the delicacy of the phosphorus test made on both fractions, one of which is known to be free of phosphorus (pseudoglobulin).

The solution of pseudoglobulin was well shaken and about 2 c.c. evaporated to dryness in a porcelain crucible. To the residue were added a small piece of solid sodium hydroxid and a few crystals of potassium nitrate, and the whole fused until a clear, white mass was obtained. After it had cooled, the crucible was half filled with distilled water and made acid to excess with a 10 percent solution of nitric acid. To a portion of this mixture, an equal volume of ammonium molybdate was added and the resultant mixture warmed. No yellow coloration or yellow precipitate was obtained. Euglobulin solution, treated similarly, gave a decided, finely divided, yellow precipitate, which settled on standing. A control test to determine the possibility of phenol interference was made with phenolized phosphate solution, and a positive result obtained. An attempt was made to test the purity of the fractions by means of precipitating serum, obtained with the respective fractions by injection into guinea-pigs. It was not possible to obtain a precipitating serum for the homologous protein. Further tests on rabbits might solve this point.

A preliminary qualitative test was made on one liter of old "serum" preserved with 0.5 percent phenol. One portion (350 c.c.) was filtered through asbestos wool under one atmosphere pressure, and 250 c.c. of the serum were fractionated at 25, 32, and 48 with saturated ammonium sulphate. The 25 percent fraction was too slight for detection. A fair precipitate was obtained with 32 percent saturation of the filtrate, and the filtrate from this fraction when made up to 48 percent yielded a heavy precipitate.

The precipitates of each fraction were separately dissolved in salt solution and the filter paper removed by straining through gauze. To each of these solutions, an equal volume of saturated ammonium sulphate was added and the fractions thus precipitated. The 32 percent fraction came down slowly. It appeared colloidal at first and, after two or three hours standing, flaked out satisfactorily. The precipitates were next treated with a volume of saturated sodium chlorid solution equal to twice that of the original volume of the serum used (i. e., 500 c.c.). The filter paper was strained through gauze and the extract allowed to stand twenty-four hours. Addition of saturated ammonium sulphate gave no precipitate. Repeated trials gave the same result. The apparently insoluble residue was dissolved in salt solution and easily precipitated with an equal amount of saturated ammonium sulphate. The explanation for this is not apparent and it seems surprising in view of the fact that Gibson¹⁵ was able to dissolve his antitoxic globulins in saturated sodium chlorid solution. It may be that the hog-cholera antiserum possesses a different chemical constitution, or that the phenol used as a preservative exerts an untoward influence over the inherent solubilities.

A second portion of this lot of serum was not filtered and was treated in the same manner to separate the protein fractions. It was found that filtration of the precipitates was retarded because of the presence of blood corpuscles and suspended corpuscular material. From a quantitative viewpoint, it would appear that the corpuscles introduce an error in the determination of the individual fractions since the primary precipitate would be unduly augmented by the

mechanical inclusion of the corpuscles. On the other hand, if the corpuscles are disrupted or dissolved, as was the case in the phenolized test serum used, the error would be slight. The method of separation just outlined was discarded in favor of the one to be subsequently presented.

The serum used for fractionating was obtained from hogs which had been immunized by means of a single injection of virus in a dose of 5 c.c. per pound body weight. The animals which showed the best temperature curve and best physical condition were bled from the tail after about ten days. The blood was collected with the usual precautions to insure sterility and was preserved with 0.5 percent phenol. The virus used for hyperimmunization was taken from such animals as showed the most characteristic lesions of hog-cholera. The antiserum was pooled from a number of hogs. This lot, known as Serial 121, was used in this work. The potency of the serum was tested by injecting, intramuscularly, varying amounts plus the virus. The test was begun Oct. 3, 1914. Animals were off test Oct. 24, 1914. The report is given below:

No. of Hog	Weight in lbs.	Virus in c.c.	Serum in c.c.	Temperature											
				10/5	10/7	10/8	10/9	10/10	10/12	10/13	10/14	10/15	10/16	10/17	10/20
1	80	2	25	102.4	102.8	102.4	103.2	103.2	102.6	102.8	102.2	102.6	102.4	103.4	102.5
2	60	2	20	102.2	102.2	102.6	104.4	104.6	103.5	103.8	102.6	103.2	103.0	102.4	103.0
3	50	2	15	102.4	102.8	102.5	107.6	107.1	106.4	108.8	105.0	102.8	102.4	103.2	103.4
4	85	2	25	102.0	102.4	101.9	103.4	104.8	104.2	103.8	103.2	103.2	103.6	102.6	101.2
5	60	2	20	101.8	101.6	102.8	103.6	103.8	102.6	103.0	102.4	103.0	103.2	103.8	102.4
6	75	2	25	102.2	102.4	102.0	103.8	104.2	103.2	103.6	103.0	102.4	102.8	102.4	104.0
7	70	2	0	102.6	102.4	102.2	105.8	106.8	Moribund, killed						
8	50	2	0	102.0	102.2	101.8	106.2	106.6	Moribund, killed						
9	85	2	0	102.4	102.6	103.2	105.6	106.4	Moribund, killed						
10	75	2	0	102.2	102.0	102.6	106.0	106.6	Moribund, killed						

From these figures, we note that 15-25 c.c. of the serum gave protection against 2 c.c. of virus. As, with one exception (Hog 3), the animals showed but slight fluctuations in temperature, it is evident that smaller amounts of serum would have sufficed to carry the animals through.

Test A.—Five hundred cubic centimeters of serum were filtered through asbestos wool under one atmosphere pressure. Two hundred and fifty cubic centimeters of the antiserum were used in the chemical separation, 100 c.c. kept at room temperature, and 100 c.c. preserved for quantitative analysis. Another lot of serum from the same stock was not filtered and was fractionated in the same manner (to be described).

In this connection might be mentioned the question of the value of red blood cells in immunization with hog-cholera serum. It was the original plan of this study to test both filtered and unfiltered serum freshly drawn from immune hogs with this end in view. At about this time, a paper appeared by Haslam and Franklin¹⁶ in which they show that the blood cells exert no protective value whatever. The authors conclude moreover that a serum from which red blood corpuscles have been removed, is definitely more potent than one containing them.

This conclusion, however, is not warranted, since injections were made with whole blood (termed "whole serum" by the writers) and centrifugated blood, no account having been taken of the actual serum volume present in each. The writers state that 0.3 c.c. of "whole serum" protected animals from hog-cholera, whereas 0.2 c.c. of the centrifugated serum were sufficient. If, as they point out, the corpuscles are inactive and comprise 40 percent of the volume of the "whole serum," then there are in reality but 0.18 c.c. of serum actually present in the amount injected. What has been shown then is merely that an added bulk of corpuscles may be dispensed with, for if the centrifugated material were more potent, less than 0.18 c.c. of serum would be necessary for a protective dose. In future work, it might be advisable to adopt uniform terms for the material injected. As used today, the so-called hog-cholera serum is in fact whole blood. Centrifugated blood or serum from clotted whole blood might be termed serum more accurately.

The method of fractionation used was as follows: 250 c.c. of the defibrinated hemolyzed blood (called "serum") (121) were precipitated with an equal volume of saturated ammonium sulphate, allowed to stand one hour (throughout the experiment the period of time allowed to elapse before filtering) and filtered. The precipitate was washed with about 100 c.c. of 50 percent ammonium sulphate solution (amount used for washing of all precipitates), pressed almost dry between filter papers, and then dissolved in approximately 300 c.c. of physiologic salt solution. This was again precipitated with the necessary amount of saturated ammonium sulphate, washed with 50 percent ammonium sulphate, and dried between filter papers. The filtrates were added to the first albumin filtrate. The globulin precipitate was dissolved in about 300 c.c. of physiologic salt solution, precipitated with a volume of saturated ammonium sulphate necessary to bring the concentration to $33\frac{1}{3}$ percent, and filtered (Euglobulin 1). The filtrate containing the pseudoglobulin fraction was measured and precipitated with a volume of saturated ammonium sulphate sufficient to bring the concentration to 50 per cent (see note). The solution was filtered and the precipitate (Pseudoglobulin 1) was dissolved in physiologic salt solution. To this was added saturated ammonium sulphate to $33\frac{1}{3}$ percent concentration. A very small precipitate was obtained. The solution was poured through the filter containing the main euglobulin precipitate. The usual volume of $33\frac{1}{3}$ percent ammonium sulphate was used in washing, and this was added to the pseudoglobulin filtrate, which was in turn brought up to 50 percent concentration with saturated ammonium sulphate, allowed to stand, and filtered (Pseudoglobulin 2). The filtrate containing albumin was added to the original albumin filtrate. The total euglobulin precipitate, pressed dry between filter papers, was dissolved in about 300 c.c. of physiologic salt solution, precipitated by one-third saturation with ammonium sulphate, and filtered (Pseudoglobulin filtrate 2). The precipitate (Euglobulin 2) was washed and the washings added to the filtrate which was freed from pseudoglobulin by bringing up the ammonium sulphate concentration to 50 percent with addition of a saturated solution. This precipitate

(Pseudoglobulin) was added to the main precipitate and the filtrate combined with the total albumin filtrates. The albumin was separated by addition of a small amount (3 c.c. per liter) of glacial acetic acid and ammonium sulphate crystals to complete saturation at room temperature. This was allowed to stand a few hours before filtering and the precipitate washed with saturated ammonium sulphate solution.

The protein fractions were suspended in salt solution and dialyzed in collo-dion sacs for four days in running water. After this time the sacs were suspended in changes of salt solution, the contents made up to the original volume of serum used, and placed in stoppered bottles.

Throughout the process, the solutions used were made up to 0.5 percent phenol concentration. In the case of the albumin precipitation, it was necessary to add a definite amount of 5 percent phenol to each lot of filtrate in order to counteract the diluting effect of the dissolved ammonium sulphate crystals. It was found that an additional 25 c.c. of phenol to each liter of albumin filtrate was sufficient to maintain a germicidal concentration when solid ammonium sulphate was added to the point of saturation. During filtration the funnels were kept covered and the filtrates collected in flasks to prevent undue change in concentration.

Test B.—A new lot of serum (250 c.c.) from the same stock (Serum 121) was fractionated without previous filtration by exactly the same method as that just outlined. The only variations to be noted are those in the volumes of the respective final filtrates obtained from precipitates and the final volumes of the separated fractions which in the test were made up to 500 c.c. with phenolized physiologic salt solution.

The amounts of saturated ammonium sulphate solution to be added in order to raise a solution to the proper concentration were calculated from the formula

$$x = \frac{v (c_2 - c_1)}{100 - c_2}$$

in which

x = number of cubic centimeters of saturated ammonium sulphate necessary to bring solution to required concentration.

v = number of cubic centimeters in original solution.

c_1 = percentage concentration (initial).

c_2 = percentage concentration (final, or desired).

QUANTITATIVE

Analyses were made of the serum to determine the percentage composition. Two methods were followed in this part of the work. The first method used was step for step like that used in the qualitative separation of the fractions to be used for injection (q.v.). The second was a modification of the Wiener¹⁷ method.

Method 1.—Fifty and thirty-five cubic centimeters of test serum were precipitated respectively by half saturation with ammonium sulphate and further fractionation carried out as described in an earlier part of the paper. The precipitates were collected on weighed, small filter papers of very fine quality (dried

in oven for one hour at 110-115 C., placed in desiccator for twenty minutes, and weighed). After thorough washing with the proper concentration of ammonium sulphate, each precipitate was placed in an electric oven at 110-115 C. for one and one-half hours in order to coagulate the proteins. Sulphates were removed by washing with boiling distilled water until tests with barium chlorid gave no further precipitate on the filtrate. The precipitates were again dried in the oven at 110-115 C. for an hour, placed in a desiccator for twenty minutes to one-half hour, and weighed rapidly. The results given below are for a test made in duplicate:

Fraction		Weight of Filter Paper in Gms.	Weight of Paper + Precipitate in Gms.	Weight of Precipitate in Gms.	Percentage Dry Weight	Average
Pseudoglobulin	A	0.4915	0.5320	0.0405	0.63	
Pseudoglobulin	B	0.5200	0.5430	0.0230	0.50	0.56
Euglobulin	A	0.5020	1.8160	1.3140	20.3	
Euglobulin	B	0.5270	1.4340	0.9070	20.0	20.15
Albumin	A	3.8750	59.8	
Albumin	B	2.5540	56.4	58.1

The percentage dry weight was calculated from the weighed residue obtained by evaporating a weighed amount of the same serum undergoing test. Five cubic centimeters of the serum were placed in a porcelain crucible and heated in an electric oven at 45 C. for fifteen hours, then placed for six hours in an electric oven at 110-115 C.

Weight of 5 c.c. serum = 4.9715 gm.

Weight of 5 c.c. serum (dried) = 0.6470 gm.

SUMMARY OF ANALYSES

	50 c.c. Serum	35 c.c. Serum
Percentage euglobulin.....	20.3	20.0
Percentage pseudoglobulin.....	0.63	0.5
Percentage albumin	59.8	56.4
Percentage of total protein.....	80.73	76.9

The total protein in the blood plasma of the pig was found by Lewinsky¹⁸ to be 80.5 percent of the whole.

Method 2.—Wiener¹⁹ has stated that the weight of protein precipitated from greatly diluted serum by one-half saturation with ammonium sulphate is always less than that precipitated from undiluted serum. He accepts the lower value as the correct one since the precipitate from undiluted serum carries with it protein belonging to other more soluble fractions and since the precipitate from diluted serum is quite insoluble in ammonium sulphate of the corresponding concentration. The precipitate from undiluted serum, furthermore, cannot be freed entirely from foreign proteins by washing with ammonium sulphate solution.

Twenty cubic centimeters of serum from the same lot were diluted to a volume of 400 c.c. with physiologic salt solution. Instead of fractionating the globulin, the total globulin was precipitated by means of half saturation with ammonium sulphate, as a check upon the method used in separating the protein

18. Pfüger's Arch. f. gesamt. Physiol. d. Mensch. u. Thiere, 1903, 100, p. 611.

19. Ztschr. f. physiol. Chem., 1910, 74, p. 29.

fractions for injection (q. v.). The precipitate was washed with 50 percent ammonium sulphate until protein-free and dried for one hour in the electric oven at 110-115 C. to coagulate the protein. The precipitate was washed with boiling distilled water until free from sulphate, dried in the oven, and weighed.

Weight of Filter Paper	Weight of Paper and Precipitate	Weight of Precipitate	Percentage of Original Protein
1.6330 gm.	2.1764 gm.	0.5434 gm.	21.0

From this result, it will be seen that the total euglobulin obtained by adding the euglobulin and pseudoglobulin fractions from undiluted serum was about the same as that obtained from diluted serum with one precipitation (see results of analysis). At first glance, this is not in harmony with Wiener's work, if we may accept this one analysis as a criterion. It is to be noted however that in the first method of separation used in this paper, the globulin was further split up and "purified," so that the fractions were free of more soluble proteins. The total globulin thus obtained is not comparable to a globulin precipitate which might be brought out of solution by a single precipitation by means of half saturation with ammonium sulphate. We have in reality an actual dilution and hence the results are essentially in accord with Wiener's findings.

IMMUNIZATION WITH PROTEIN FRACTIONS FROM HCG-CHOLERA SERUM

Six hogs were injected with euglobulin, pseudoglobulin, and albumin fractions, respectively, plus virus. Each animal received 2 c.c. of virus simultaneously with the serum. Intramuscular injections were made.

No. of Hog	Weight lbs.	Virus c.c.	Serum c.c.	Fraction *	Result
858	70	2	25	Whole serum	Well.
1606	80	2	25	Whole serum	Well.
1967	70	2	50	Euglobulin	Died. <i>Bacillus suis septicus</i> found in pure culture.
1156	65	2	50	Euglobulin	Well.
1481	60	2	50	Pseudoglobulin	Well.
982	60	2	50	Pseudoglobulin	Well.
1592	60	2	Bled. Typical cholera lesions.
1164	60	2	Bled. Typical cholera lesions.
900	60	2	50	Albumin	No protection. Killed. Typi- cal cholera lesions. + + +
901	70	2	50	Albumin	No protection. Killed. Typi- cal cholera lesions. + +

* The fractions were made up to double the volume of original serum.

A review of this result shows that both the euglobulin and pseudoglobulin fractions of the serum possess marked protective value. In other words, the globulin fractions, as a whole, contain the antibodies. The animals, with exception of the "virus" and "albumin" hogs, and one hog treated with euglobulin, were never off feed. The last named was chronically ill and died after two weeks. Some protection was given by the fraction injected, but secondary complications appeared to have set in. Postmortem lesions were typical of cholera and *Bacillus suisepicus* was found in all organs. Intraperitoneal doses of 0.5 c.c. of an agar slant emulsion made from these cultures killed guinea-pigs in two to three days.

The protective value of these fractions, taken together, is about five pounds for each cubic centimeter of serum. Comparison with the potency of whole serum renders this figure somewhat inconsistent. The whole serum, as shown in Tables 1 and 2, gave about three pounds protection for each cubic centimeter. However, since there were but slight, if any, untoward symptoms in these test animals, it is very likely that a smaller amount of serum would have protected as well as the fractional serum. In that event, the protective value, expressed in pounds body weight, would be about the same. Moreover, some of the animals treated with the serum fractions ran close to the margin, as may be seen from the temperature chart. Administration of somewhat larger doses would of necessity have lowered the figures for protective value per pound and thus have approximated more nearly the value in terms of unfractionated serum. Because of a scarcity of material, it was not possible to run detailed quantitative experiments. It may be of interest to determine by varying doses whether or not the fractions possess different degrees of protective power. The quantitative determinations made on the serum point to the fact that the pseudoglobulin fraction is considerably more potent than the euglobulin. The latter constitutes about 20 percent of the total protein whereas the former represents but 0.5 percent, being thus forty times more effective. With such a small series, however, these figures must not be regarded as conclusive, even if we were to overlook the very important factor of idiosyncrasies in individual animals. Differences in antibody content may be due to a number of factors. As has been intimated in the chemical separation, a loss may occur in the lower fraction due to its solubility in the higher concentration of ammonium sulphate. Any relative differences in the distri-

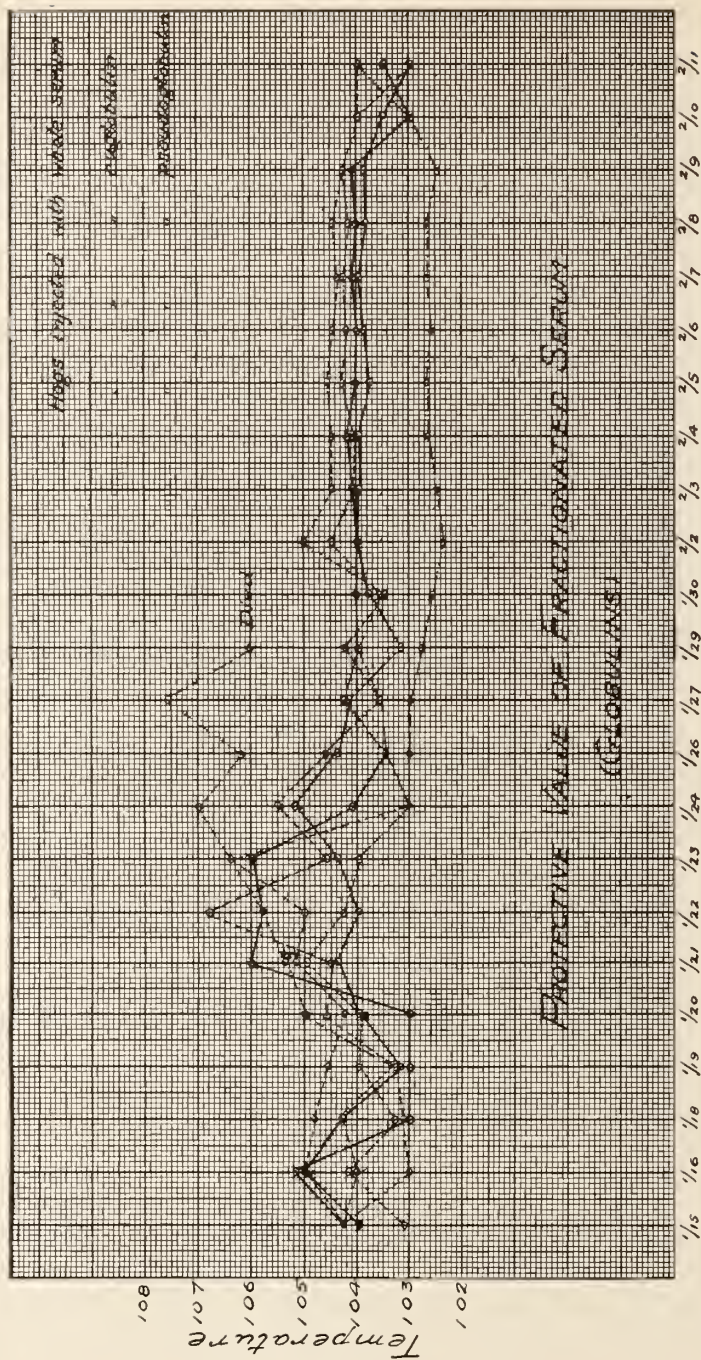


Chart 1.—The globulin fraction here shows protection. The animals with one exception were protected. This one at autopsy, as noted elsewhere, showed complications.

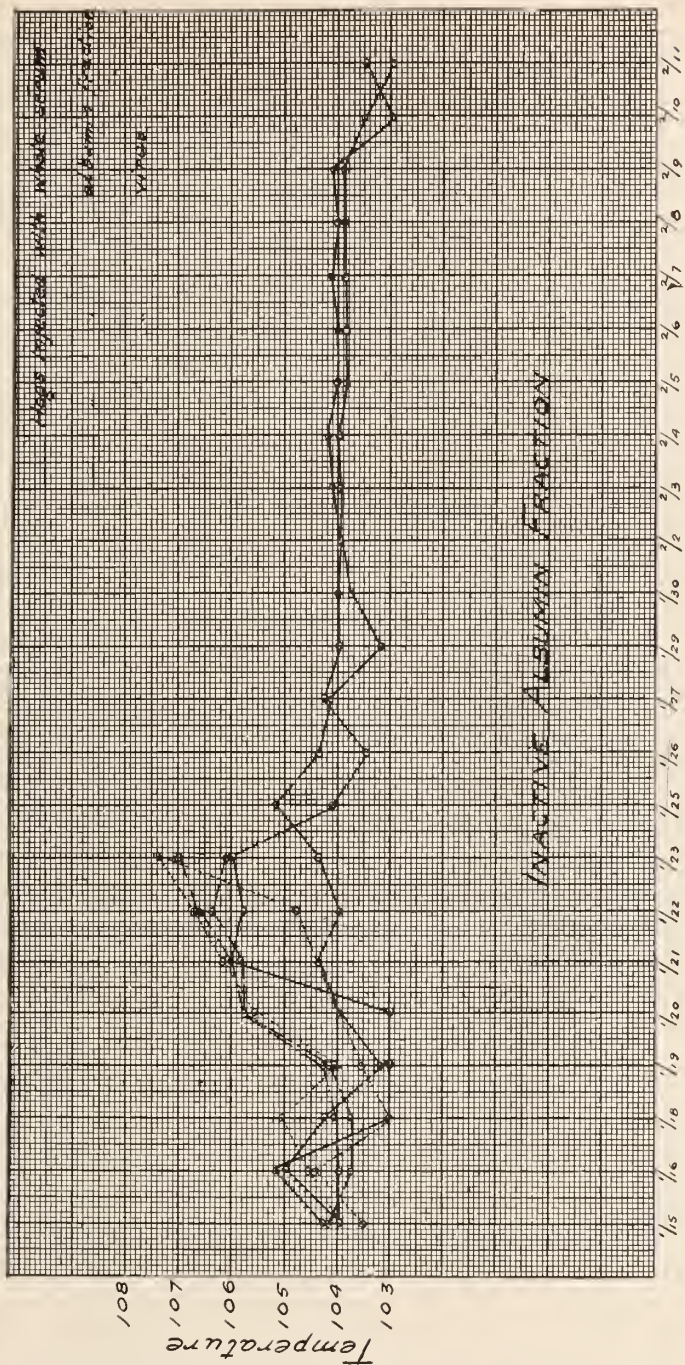


Chart 2.—The albumin fraction does not exert any protection. The temperature curve follows closely that observed in the case of hogs treated with virus alone.

bution of antibodies should be more pronounced for the lower fraction. This has been shown by Gibson and Collins²⁰ in their work on diphtheria antitoxin. It is not likely that this phase enters here in so striking a manner, since the material injected was not in concentrated form and since the possible inclusion of a lower fraction in the higher would not be sufficiently great to alter the protective value to any extent.

The albumin fraction is entirely inactive. On the seventh day, the animals ran a high temperature and were very sick. Death was imminent on the eighth day. A comparison between the temperature curves of these animals and those of the controls (virus alone) will show a striking similarity.

The charts here given show graphically the relative value of the serum fractions. Chart 1 represents the effectiveness of the split globulins as compared with the unfractionated serum. In Chart 2 are given the results obtained with albumin separated from hog-cholera antiserum.

SUMMARY AND CONCLUSIONS

Hog-cholera serum can be split up by chemical means into an actively protecting globulin fraction and an inactive albumin fraction.

Precipitation of serum proteins by means of ammonium sulphate is practically possible for hog-cholera serum. The bulk of the serum, being inactive albumin, may be dispensed with.

Concentration for practical purposes may be effected (1) by precipitating the euglobulins from diluted serum, by means of $33\frac{1}{3}$ percent saturation with ammonium sulphate solution, filtering, making the filtrate up to 50 percent concentration with ammonium sulphate solution, filtering, and after dialyzing the precipitate in running water, dissolving it in the smallest volume of salt solution; (2) by precipitating the diluted serum (diluted 10-15 times) by one-half saturation with ammonium sulphate (saturated solution), filtering, dialyzing the precipitate, and treating as in (1). Since both globulin constituents are protective, this method would prove more economical and simpler.

Euglobulin represents from 20-21 percent of the total serum protein, pseudoglobulin 0.5 percent, and albumin about 80 percent.

20. Jour. Biol. Chem., 1907, 3, p. 233.

TOXINS AND THE SIDE-CHAIN THEORY *

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The toxins are distinguished from other poisonous agents by their property of stimulating the production of antibodies (antitoxins) when they are injected into animals. While, previous to the studies on cobra venom, nothing had been known regarding the mechanism of the action of toxins, that action had found an attempted explanation in the widely accepted side-chain theory of Ehrlich,¹ which considers not only the poisonous action of toxins, but also the mechanism of antitoxin production.

According to the side-chain theory, the toxin molecule is conceived to possess a chemical group by which it is capable of entering into firm¹ chemical union, through a corresponding chemical group, with the sensitive tissue. The uniting group of the toxin molecule has been named the "haptophore" group and that of the vulnerable cell, "receptor." The union of toxin and cell having taken place—but not till then—the toxin is able to inflict its characteristic injury upon the cell, that injury being produced by a second chemical group, the so-called toxophore group. The tissue receptors that have united with the introduced toxin are assumed to have been thrown out of function in the cell economy and this elimination of the "receptors" is supposed to constitute in itself an injury that must be repaired. The injury caused by the mere union of the "haptophore" group with the cell receptors is different from that subsequently inflicted by the "toxophore" group, since the former injury must also be assumed, under the side-chain theory, to follow the injection of non-toxic antigenic substances, such as foreign blood sera or corpuscles. In repairing the defect, the cell is believed to produce a greater number of the "receptors" than were used up by the toxin. The excessive "receptors" are thrown off into the body fluids and constitute there the specific antibodies, or antitoxin. In explaining this excessive production of the "receptors," Ehrlich has cited the analogous excessive production of tissue in the repair of the

* Received for publication June 8, 1915.

1. Proc. Roy. Soc., 1900, 66, p. 424.

loss of substance following injuries. This parallel clearly expresses the conception that the union of haptophore group and cell receptor results in injury to the cell, for there is in nature no known instance of a loss of tissue not accompanied with injury that is followed by an overproduction of that tissue.

It should be remarked here in passing that the laws governing the response of the cells to antigenic stimulation are quite different from those controlling the response of tissues to the effect of injurious agents; that is, to irritants. It is a fact of common knowledge that if a tissue be exposed at intervals to the influence of an irritant, that tissue becomes increasingly resistant to the irritant and the cell multiplication, which is the index of reaction on the part of the tissue, becomes less and less. In their discussion of this point, von Dungern and Werner² write:

If a definite irritation be repeated at intervals of sufficient length, so that in the interim the cells may return to the resting state, an important phenomenon will be observed. The reaction to the irritant becomes less and less, until finally the same degree of irritation that at first set up a vigorous growth is no longer able to produce any increase in proliferation. If an irritant be applied continuously or at very short intervals, the end result is different according to the intensity of the irritation. If the latter be mild, an increased power of resistance against the growth-producing irritant takes place, so that the proliferation not only does not progress, but at last actually diminishes. If, on the other hand, the intensity of the irritant is great, the recovery of the cells from the injury is prevented, and, as a result of a cumulative action of the irritant, an increase of growth takes place up to a definite maximum, to be followed, upon further irritation, by the death of the cell.

On the other hand, tissues that are exposed at intervals to antigenic stimulation exhibit, at first, more and more sensitiveness to that stimulation, as is shown by the increased production of the specific antibodies, up to a certain height, after which, instead of a power of resistance, or hyposensitiveness, developing against the antigenic stimulation, the response may continue unabated for an indefinite period, subject only to the condition of the general health, which, it must be remembered, may be affected by the injections of the antigen.

The effect of repeated injections carried out over an extended period has been exactly studied by Elser and Huntoon, to whom I am greatly indebted for permission to refer to some of those studies in advance of their final publication: Four rabbits, 441, 234, 286, and 285, received daily intravenous injections of the same small amount of

meningococci over periods of 13 months, 12 months, 8 months, and 36 months, respectively (the last animal receiving 1091 injections). In all of these animals, the rate of antibody production increased rapidly, at first, up to a maximum, after which, with the exception of certain fluctuations of greater or less extent, which often could be referred to conditions affecting the general health of the animal, such as pregnancy and infection, the rate remained relatively high. In no instance was a decline observed in the rate of antibody production that could be referred to a repetition of the injections themselves. Two other rabbits that received similar injections of typhoid bacilli over a period of three months responded in precisely the same way.

Two rabbits that I have treated in the same manner with sheep blood corpuscles (0.1 c.c. of blood daily) yielded a hemolytic serum of a strength of 1:40,000, which was maintained practically unchanged for a period of several weeks.

It is seen that the reaction of the tissues to antigenic stimulation is different from that of tissues to injurious irritation. This antithesis, which hitherto has been overlooked, cannot be compromised; it prevents the application of the principle of Weigert to the explanation of the mechanism of antibody production. The reaction of the tissues to antigenic stimulation finds a more plausible physiologic analogy in the response of digestive glands to specific chemical stimulation offered by foodstuffs than in the response of tissues to injurious influences.

The toxins bear a long-recognized, striking resemblance to the organic ferments. This resemblance, however, has not led to any general belief in the fermentative nature of the toxins. On the contrary, the only writer on this question in recent times, von Liebermann,³ emphatically denied that the toxins are ferments. Moreover, von Liebermann made the noteworthy remark that the establishing of the fermentative nature of the toxins would render the side-chain theory untenable. Von Liebermann based his denial of the fermentative nature of the toxins upon the results of his experiments with the agglutinating property of ricin and abrin. Those results are as follows: (1) The agglutinating principle of these preparations is quickly absorbed in a definite quantitative proportion by the blood corpuscles; that is, these "toxic" bodies are "used up" in the reaction, whereas the ferments, theoretically, are not used up. (2) The ricin agglutinin is

3. Deutsch. med. Wchnschr., 1905, 31, p. 1301.

not appreciably affected by hydrocyanic acid, which is a "violent enzyme poison." (3) The agglutinating property of ricin is not destroyed by exposure for half an hour to a temperature of from 70 to 80 C.; that is, this "toxic" body is not thermolabile as are the ferments. Von Liebermann recognized that the incompleteness of our knowledge about ferments could be urged against drawing any deductions regarding the fermentative nature of toxins, but believed nevertheless that that knowledge was sufficient to support the evidence on which he based his denial. However, it is just at that point that his whole argument has fallen; for since he published these views a ferment has been found that is absorbed by the substratum upon which it acts (appearing thus to have been used up), that is not appreciably affected, as we shall show, by hydrocyanic acid, and that is not affected by exposure for half an hour to a temperature of over 70 C. The ferment referred to is the lipase of cobra venom, which is also the hemotoxin of the venom.

The absorption of the lipase of cobra venom by lecithin was first demonstrated by Kyes and amply confirmed by von Dungern and Coca, and Manwaring. Von Dungern and Coca showed that the absorbed lipase was not actually used up, but could be demonstrated in active condition in the digestion products of the lecithin, and later Manwaring was able to isolate the lipase again from those digestion products.

Therefore, the fact that a toxin is absorbed by the vulnerable tissue does not show that the toxin is used up in the process of its activity; hence, its ferment nature cannot be disputed on this ground.

We have investigated the influence of hydrocyanic acid upon the lipase of cobra venom and have found that the lipolytic action of the ferment is not interfered with by this acid.

Six and one-half grams of potassium cyanid (Kahlbaum, 96-98 percent pure) were mixed with 47 c.c. of strong HCl (10 c.c.-2.9 c.c. normal), and the final volume of the mixture was then brought up to 50 c.c. by the addition of distilled water. The resulting fluid thus contained about 5.2 percent of hydrocyanic acid and 15 percent of potassium chlorid. The reaction of the fluid was very slightly alkaline to litmus, and twenty drops of it caused no hemolysis of 1 c.c. of a 5 percent suspension of ox blood corpuscles.

Four dilutions of cobra venom (for which I am under obligation to Prof. A. Calmette, director of the Pasteur Institute in Lille), were prepared containing, respectively, 1 in 10,000, 1 in 100,000, 1 in 1,000,000, and 1 in 10,000,000 of the venom. Three series of twelve tubes each were arranged, each series containing 1 c.c., 0.5 c.c., and 0.2 c.c. of the four venom dilutions, and each tube containing 0.1 c.c. of a 0.2 percent emulsion of lecithin, which by itself was not hemolytic in a quantity of 0.4 c.c.

To each tube of Series A was added 1 c.c. of the hydrocyanic acid solution (von Liebermann used 0.2 c.c. of a 72 percent solution) and to each tube of Series B was added an equal volume of a 15 percent solution of potassium chlorid. No addition was made to the third series, C.

After these three series had stood for twenty-four hours at room temperature, 1 c.c. of a 5 percent suspension of ox blood was added to each tube and the hemolysis resulting at room temperature was observed. In the first three tubes of Series C, hemolysis was complete in a few minutes, whereas in the corresponding tubes of Series A and B complete solution occurred much later (from one to three hours). At the end of a further twenty-four hours, the tube of Series C containing 1 c.c. of the fourth dilution showed complete hemolysis; that containing 0.5 c.c. of the fourth dilution showed strong hemolysis; and that containing 0.2 c.c. of the fourth dilution showed a trace of hemolysis. In Series A, the corresponding tubes showed complete hemolysis, slight hemolysis, and a trace of hemolysis. In Series B, the corresponding tubes showed strong hemolysis, no hemolysis, and no hemolysis. It is seen that not even during twenty-four hours contact was the hydrocyanic acid able appreciably to interfere with the lipolytic activity of the venom.

The relative resistance of the cobra venom lipase to heat is already known. Heating a 1 percent solution of the venom for three quarters of an hour at 72 C. does not noticeably affect its lipolytic activity, which is undiminished even by short boiling.

All of the arguments brought by von Liebermann against the theory of the fermentative nature of the toxins are thus seen to have lost their force through the subsequent studies on the lipase of cobra venom. Moreover, these same studies have demonstrated that one toxin (the hemotoxin of the venom) is actually a ferment; in other words, the hemotoxin has been shown to be identical with the lipase of the venom.

The hemotoxin of cobra venom is that constituent of the venom which destroys red blood corpuscles. Not all species of red blood corpuscles are susceptible to the direct action of the hemotoxin; those of the guinea-pig and man, for example, being very susceptible, and those of the ox and the sheep being non-susceptible. The red blood corpuscles that are invulnerable to the direct action of the hemotoxin can be made vulnerable with the co-operation of certain "activators," such as normal serum and lecithin. The mechanism of the "activation" is not the same with these two agents. The destruction of the corpuscles is brought about in the two cases through the action of two different constituents of the venom. This is shown by the fact that, on the one hand, treatment of the normal serum in the cold with the invulnerable corpuscles greatly diminishes the activating power of the serum, and that, on the other hand, heating the venom solution in an

acid medium causes it to lose its power of destroying the invulnerable corpuscles when it is mixed with normal serum, altho it is still able to do so in co-operation with lecithin.

The present discussion concerns itself only with the venom constituent that attacks the naturally invulnerable corpuscles with the aid of lecithin. The researches of Kyes, Kyes and Sachs, von Dungern and Coca, and Manwaring have shown that the venom-lecithin hemolysis is brought about by the fermentative action of the venom on the lecithin whereby the latter, a non-hemolytic substance, is split into two parts both of which are strongly hemolytic. These two parts are oleic acid and the lecithin rest, which is lecithin in which one of the two fatty acid molecules is missing. Because of the demonstrated fact that this mono-fatty acid lecithin rest contains no oleic acid, it has been designated "desoleolecithin." The properties by which this substance is recognized are, its solubility in alcohol and water, its insolubility in ether, and its great hemolytic power.

It has been assumed that the mechanism of the direct hemolytic action of the cobra hemotoxin in destroying naturally vulnerable corpuscles is the same as that of its indirect hemolytic action in co-operation with lecithin. The immediate hemolytic agents in the former case would thus be the split products of the lecithin that is normally present in the red blood corpuscles in a quantity sufficient to supply a completely hemolytic amount of desoleolecithin and oleic acid. In accordance with this assumption, it was conceivable that desoleolecithin could be demonstrated in the fluid resulting from the direct hemolytic action of the venom on naturally vulnerable corpuscles. The following experiments will show that such demonstration is possible.

A preliminary experiment was carried out in order to find out what part of a definite quantity of desoleolecithin that has been mixed with blood corpuscles can be recovered with the method at our disposal.

One hundred cubic centimeters of ox blood were well washed with physiologic salt solution and the corpuscular sediment was mixed with 0.2 gm. of desoleolecithin (two complete hemolytic doses) which had been dissolved in salt solution. (The minimal completely hemolytic quantity of this preparation for 1 c.c. of 5 percent ox blood was 1/20,000 gm.) After the resulting hemolysis was complete, 200 c.c. of distilled water and 100 c.c. of 95 percent alcohol were added and the mixture was boiled for a few minutes. The fluid, which will be referred to as the "first extract," was separated from the coagulated proteins by filtration. The coagulated proteins were mixed with 400 c.c. of 95 percent alcohol and allowed to stand over night at room temperature.

The first extract was evaporated at first by boiling and finally with the use of an electric fan, and the residue was extracted with ether. The ether extract

was discarded and the residue was again extracted with warm absolute alcohol. The alcoholic extract was evaporated with the use of the electric fan, and the residue was taken up in 20 c.c. of physiologic salt solution. One cubic centimeter of a 5 percent suspension of washed ox blood was completely hemolysed in a few minutes by 0.4 c.c. of this solution and slightly dissolved by 0.2 c.c. of the solution. The entire 20 c.c. therefore contained enough of the hemolysin to dissolve completely 2.5 c.c. of undiluted ox blood.

The second alcoholic extract of the coagulated proteins of the ox blood was obtained by filtration after the mixture of the proteins and after the alcohol had stood over night at room temperature. The filtrate was evaporated to dryness with the use of the electric fan, and the residue thus obtained was extracted with ether, the ethereal extract being discarded. The residue was then extracted with warm absolute alcohol and the alcoholic extract, separated by centrifugation, was evaporated to dryness and the resulting residue taken up in 10 c.c. of warm physiologic salt solution. This solution hemolysed 1 c.c. of 5 percent ox blood in a minimal quantity of 0.02 c.c., and the entire 10 c.c. contained, therefore, enough of the hemolysin to dissolve completely 12.5 c.c. of undiluted ox blood.

The hemolysin obtained by the procedure just described was identified as desoleolecithin, first, by its solubility in alcohol and water, and its insolubility in ether, which distinguishes it from the hemolytic substances in the extracts of normal tissue; secondly, by the rapidity of its hemolytic action, the minimal dose producing complete hemolysis within a few minutes; and finally, by reason of the failure of this method of extraction, in several experiments, to demonstrate any such hemolysin in corpuscles that had not been dissolved either with cobra venom or desoleolecithin. The experiment therefore demonstrates that even if a quantity of desoleolecithin capable of completely dissolving 200 c.c. of undiluted ox blood be mixed with only 100 c.c. of such blood, it is possible, with the method of extraction used, to recover from the hemolyzed corpuscles only about 7.5 percent of the desoleolecithin taken. By far the greater part of the hemolysin was combined with the corpuscular substance in such a way that the method of extraction employed failed to separate it.

The same method of extraction was applied to guinea-pig blood corpuscles that had been hemolyzed either with distilled water or with native cobra venom. In the former instance no hemolytic substances at all were obtained, but in the latter instance a quantity of hemolysin having the properties of desoleolecithin previously mentioned was obtained that was equivalent to about 2.5 percent of the calculated minimal amount necessary completely to dissolve the corpuscles used.

For this experiment 400 c.c. of defibrinated guinea-pig blood were well washed with physiologic salt solution and the corpuscular sediment was divided into two equal portions. To one portion was added 0.1 gm. of cobra

venom dissolved in 5 c.c. of physiologic salt solution. After three quarters of an hour at room temperature, microscopic examination showed that the corpuscular forms had completely disappeared and that the thick fluid contained masses of hemoglobin crystals. At the end of two hours, 100 c.c. of distilled water were added to each portion of blood and both portions were treated according to the method of extraction previously described. Thirty cubic centimeters of the final salt solution extract were obtained from each portion. That derived from the blood that had been dissolved with distilled water lacked completely any hemolytic power; that derived from the portion of blood that had been dissolved with cobra venom was completely hemolytic in a minimal quantity of 0.3 c.c.

The entire amount of the extract thus contained enough of the hemolysin to dissolve 100 c.c. of 5 percent guinea-pig blood, or 5 c.c. of undiluted blood, which is 2.5 percent of the quantity in each portion of blood used for the experiment.

A similar experiment, the notes of which have been lost, led to the same result; a considerable quantity of the hemolysin possessing the properties of desoleolecithin was again extracted from guinea-pig corpuscles that had been dissolved by the direct action of cobra venom.

The same method of extraction was applied also to ox blood corpuscles that had been dissolved by the combined action of anti-ox corpuscle amboceptor serum and normal guinea-pig serum, with negative result, the method of extraction failing to discover the production in the dissolved corpuscles of any hemolytic substance possessing the properties of desoleolecithin.

The sediment of 200 c.c. of well-washed ox blood was mixed with 25 c.c. of the serum of a rabbit that had received injections of ox blood, and to this mixture were then added 200 c.c. of normal guinea-pig serum. Two hours later, complete hemolysis having taken place in thirty-five minutes, the mixture was treated with the alcohol and ether extraction method. The final residue was taken up in 3 c.c. of salt solution, and this solution was then found to be entirely lacking in hemolytic activity when mixed with 1 c.c. of 5 percent ox blood.

The results of the experiments just described leave no room for doubt as to the mechanism of the direct destructive action of the cobra hemotoxin. It is clear that this hemolytic action is brought about by the lipolytic power of that constituent of the venom whereby the normally contained lecithin, and no doubt other fatty substances as well, are split into products that are the ultimate cell-destroying substances. It follows, therefore, that the cell destruction in this instance cannot be due to any assumed chemical group (toxophore group) in the toxin molecule.

Furthermore, if any of the toxin does attach itself to the cell proper according to the conception of the side-chain theory (through a haptophore group), as has been shown by von Dungern and Coca⁴ to occur when naturally immune corpuscles (ox corpuscles) are brought into contact with a solution of cobra venom, the cell is not injured thereby. It is only when the toxin reaches the lecithin, becomes dissolved in it, and splits it into desoleolecithin and oleic acid, both of which are highly injurious to the cell, or when it reaches some other fatty substance and splits it into glycerin and some lower fatty acid, both of which are cell poisons—only then does the injurious influence of the hemotoxin reveal itself.

The two important results that have come out of the investigations of the hemotoxin of cobra venom are: First, the demonstration of the fermentative nature of a toxin, and second, the demonstrated failure of the side-chain theory in the first real test to which it has been put as an explanation of the mechanism of toxin action. In view of the first of these results, it seems safe to assume that all toxins may be ferments. The sources of the ferments and those of the toxins are the same. Ferments as well as toxins are known to exist in glandular secretions of animals and in the products of the growth of bacteria and in the higher forms of plant life. We have already recalled the long-recognized similarity between the general properties of ferments and those of toxins.

Another result of the investigations of the hemotoxin of cobra venom is the explanation of the natural resistance of the invulnerable corpuscles (ox and sheep) to the direct action of the hemotoxin. This natural resistance, or immunity, has been found to depend upon a physical condition of the cell substance that prevents the hemotoxin from penetrating to the lipoids of the corpuscles. This was shown by the discovery of Goebel⁵ that the mere suspending of the naturally immune corpuscles in a chemically inert solution of sugar suffices so to alter the physical condition of the corpuscular substance that the hemotoxin can then enter the cells, reach the lipoids, and cause hemolysis. Other substances, also, such as soap and oleic acid, have been found to produce a physical alteration of the corpuscles that is similar in effect to that produced by the sugar.

As the discovery of the nature and manner of action of the hemotoxin of cobra venom has revealed the probable nature of the other

4. München. med. Wchnschr., 1907, 47, p. 2317.

5. Compt. rend. de Soc. de biol., 1905, 58, p. 422.

toxins, so also the explanation of the natural immunity of the invulnerable corpuscles to the direct action of the venom hemotoxin may provide a clue to the mechanism of some of the other known instances of natural immunity to toxins.

It is evident that the explanation offered by Ehrlich¹ for natural immunity cannot be brought into harmony with the demonstrated mechanism of the natural immunity of blood corpuscles to the direct action of the hemotoxin of cobra venom. Ehrlich's explanation was that the cells of the naturally immune animal do not possess receptors capable of entering into chemical union with the haptophore group of the respective toxin molecule. This explanation is incompatible with the demonstration of Goebel that the natural immunity of invulnerable corpuscles to the direct action of cobra hemotoxin is dependent upon a physical condition of the corpuscular substance.

Of the numerous cases of natural immunity to toxins that have been studied, only few are known (such as that of the scorpion to its own venom and that of the hedge-hog to the viper's venom) which could be referred to any antitoxic power of the blood. Indeed, some of the naturally immune animals are not capable of producing antitoxic substances, even after having received large injections of the toxin.

There are a few cases known in which a relative natural immunity is present so long as the body temperature of the animal remains below a certain point. The frog, in winter, and the hibernating bat and marmot possess such a relative resistance to tetanus toxin. At the higher "summer" temperature, all these animals become susceptible.

The remarkable natural immunity of the fowl against tetanus toxin remains an inexplicable phenomenon. The blood of the fowl is entirely lacking in antitoxic property; the immunity is therefore not humoral. Under ordinary condition of health, large subcutaneous or intraperitoneal injections of the toxin are borne by fowl without symptoms; but, if the injections are made into the brain or after the fowl have become weakened by cold, the toxin is able to produce its characteristic effect. It is conceivable that in this case, as well as in a number of other instances in which the immunity is demonstrably not humoral, the physical condition of some protective tissue or of the sensitive cells themselves plays a determining role.

A RAPID AND EFFICIENT METHOD OF PRODUCING HEMOLYTIC AMBOCEPTOR AGAINST SHEEP CORPUSCLES *

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From the time when the principle of complement-deviation was applied to the serum diagnosis of disease, as in syphilis, gonococcic infection, glanders, and malignant tumor, the possession of a "good" hemolytic immune-serum, usually prepared against sheep corpuscles, became a necessity to the clinical laboratory. By a "good" hemolytic serum is meant one that possesses a sufficiently high hemolytic strength, which must remain permanently constant.

The usual method of producing hemolytic sera has been to inject relatively large and increasing quantities (5-20 c.c., in some laboratories even as much as 50 or 75 c.c.) of the washed corpuscles at intervals varying from three days to one week, until four or five injections have been given, the bleeding of the animals taking place about one week after the last injection. The results obtained by this procedure have been often unsatisfactory, it being almost exceptional for the hemolytic serum thus produced to possess both of the requirements mentioned.

Following the publication by Fornet and Muller¹ of experiments showing that precipitating sera of high potency can be produced by injecting the antigen in increasing amounts on three successive days, this rapid method of immunization was applied by Bonhoff and Tsuzuki,² without success, to the production of hemolytic sera against the corpuscles of the sheep, the horse, man, and the pig. The titer of the resulting sera was uniformly low, 1:10.

More recently Gay and Fitzgerald³ repeated these experiments of Bonhoff and Tsuzuki, using only sheep blood corpuscles and injecting quantities of 1 or 2 c.c. intravenously. According to Gay and Fitzgerald, such injections, administered on three successive days, were fol-

* Received for publication June 8, 1915.

1. Ztschr. f. biol. Technik u. Methodik., 1908, 1, p. 201.

2. Ztschr. f. Immunitätsforschung, 1910, 4, p. 180.

3. University of California Publications in Pathology, 1912, 2, p. 79.

lowed by the development of an unusually strong hemolytic property in each of the eight animals used for the experiment.

For a number of years, the writer has studied the production of hemolytic sera in rabbits and has been able, on the basis of many observations, to formulate some generalizations that are of practical value to routine serology.

1. The first of these generalizations is the well-known fact that the individual rabbits differ greatly in their response to the injection of the foreign corpuscles. These individual differences, which are both quantitative and qualitative, are illustrated in the following experiment:

Six normal rabbits received, on June 16, 2 c.c. each of washed sheep corpuscles, intravenously injected, and on June 21 a second intraperitoneal injection of 10 c.c. each. On June 30, the sera of the rabbits were examined as to their hemolytic activity. In these tests, as in all the subsequent tests of hemolytic strength referred to in this communication, the different reagents were combined in one-tenth of the usual quantities, i. e., the different hemolytic sera were first diluted 1:10 and then mixed in descending amounts with 0.1 c.c. of a 5 percent suspension of washed sheep corpuscles (based on the volume of full blood taken and representing about 2.5 percent of corpuscular sediment) and 0.1 c.c. of a 1:10 dilution of fresh guinea-pig serum. The smallest quantity of the diluted hemolytic serum that caused complete hemolysis of the sheep corpuscles was noted as indicating the relative hemolytic strength of the serum.

The results of the test were:

In Rabbits 11, 12, 15, and 16.....	0.0002
In Rabbit 10	0.0004
In Rabbit 9	0.0008

Five weeks later, the sera that had been obtained from Rabbits 11, 12, 15, and 16 on June 30 and preserved by the addition of 0.25 percent of carbolic acid, were tested again. The results of this test with Sera 11, 15, and 16 were identical with those obtained at the first examination; whereas the titer of Serum 12 had fallen to about 0.0008, i. e., to about one-fourth of its original strength.

It is seen that the same previous treatment in the six rabbits resulted in a high hemolytic potency in only four of the animals, and that, of these four, only three furnished hemolysins that were entirely stable.

2. It has been found that the quality of the hemolysins produced by means of many injections is often different (as judged by the criteria of stability and anti-complementary property) from those obtained after few injections.

This qualitative difference was clearly seen when the immunization of Rabbits 11, 15, and 16 was resumed, as follows:

From August 9 until August 16, inclusive, daily intravenous injections of 0.2 c.c. of washed sheep corpuscles; August 25, 27, and 29 similar injections; September 23, 25, and 26 similar injections of 1.0 c.c. On October 2—six days after the last injection—the sera of the three animals were examined; they were all found to possess the same hemolytic potency, namely, 0.0001. It was observed that when the larger quantities of the hemolytic sera (0.002 c.c. or more) were combined with the unit of sheep corpuscles, if complement were immediately added, hemolysis failed to occur; whereas, if the addition of complement was deferred for about fifteen minutes, solution of the corpuscles then followed. This phenomenon has never been noticed in testing hemolytic sera that had been obtained after few injections of the corpuscles. One-quarter of 1 percent of carbolic acid was mixed with the sera and after three weeks, during which the sera had been kept in the ice-box, they were again examined; it was found that all three of them had lost over 90 percent of their original strength. The titers were: Serum 11, 0.0015, Serum 15, 0.0014, and Serum 16, 0.0012. There was no bacterial growth in any of the sera. After another interval of one month, Sera 15 and 16 were found to have exactly the same hemolytic power as at the last examination. Serum 11 had been lost by accident.

These experiences, confirmed in a number of other rabbits that had had similar treatment, show that after many injections made over a relatively long period of time the resulting hemolytic property of the serum is often in large part—over 90 percent—unstable; whereas after few injections—two in the present instance—the same animals usually yield sera the hemolytic potency of which remains entirely constant; they show, furthermore, that hemolytic sera obtained after many injections of the corpuscles possess certain anticomplementary properties, which are not found in such sera obtained after few injections.

3. It was found that the maximal degree of immunity can be effected by injecting relatively small quantities of the blood corpuscles.

The most powerful hemolytic sera that we have examined were prepared by giving daily intravenous injections of as little as 0.1 c.c. of washed sheep corpuscles for a period of many weeks. Such sera have been hemolytic in quantities of 0.00005 and 0.000025 c.c. A hemolytic serum of equally high strength has been obtained by Dr. L'Esperance with the same procedure.

We have already demonstrated (under 2) that the important quality of stability of hemolytic strength combined with high pontency can be secured only by the administration of few injections, and, under this condition, as the following experiment shows, the maximal effect

cannot be produced with so small an amount as 0.1 c.c.* One cubic centimeter, however, was found to be sufficient.

Six normal rabbits that had received two intravenous injections of 0.1 c.c. of washed sheep corpuscles at intervals of four to six days yielded hemolytic sera of an average strength of 0.001. Four other normal rabbits that had received two similar injections of 1.0 c.c. each at an interval of five days yielded hemolytic sera of an average strength four and one-half times as great—0.00022. Nine additional normal rabbits receiving primary injections of 1 or 2 c.c. and second injections of 5 or 10 c.c. yielded sera possessing an average potency of 0.00025.

From the results of this experiment it is seen that no greater hemolytic activity is obtained by injecting 5 or 10 c.c. than by injecting 1 c.c. of the corpuscles.

4. It has been found that the optimal time for making the second injection of the sheep corpuscles is not earlier than at the end of an interval of three days after the first injection; that is, on the fourth day of the treatment.

The considerable advantage obtained by making the second injection after an interval of not less than three days is graphically shown in the accompanying table. The height of the black columns represents the average relative concentration of the hemolytic substances in the blood after the different immunizing procedures.

It is seen that the low average hemolytic power resulting from a single injection is considerably increased by further injections undertaken on the second and third days—Columns 2 and 3. This increase, however, is much greater if the second injection is deferred till the fifth day—Column 4.





5. The height of the hemolysin production following the first injection of sheep corpuscles is reached after an interval of seven days, and following the second injection the new height is reached after a further interval of not more than five days.

In the accompanying chart is shown the course of the hemolysin production in six rabbits that received two intravenous injections each

* This statement applies only when more than a single injection are given, as the following experience shows: Ten normal rabbits received 0.1 c.c. each of washed sheep blood corpuscles, intravenously administered, and one week later these animals yielded hemolytic sera of an average strength of 0.004. Three other normal rabbits that had received similar injections of 2.0 c.c., that is, twenty times as much as the former series, yielded an average hemolytic power of only 0.0065. This average would, no doubt, have been higher if a larger number of animals had been used; however, it is safe to assume that even then the average hemolytic power would not have been greater than that obtained in the series in which 0.1 c.c. of the corpuscles was injected.

TABLE 1

THE ADVANTAGE OF A FOUR-DAY INTERVAL BETWEEN THE INJECTIONS OVER A TWO-DAY INTERVAL AND THE SO-CALLED "RAPID METHOD"—COLUMN 3—IN THE IMMUNIZATION OF RABBITS AGAINST SHEEP CORPUSCLES

AVERAGE TITER OF			
Thirteen Rabbits Receiving a Single Injection	Three Rabbits Receiving Two Injections, the Second Injection being Given on the Third Day	Four Rabbits Receiving Injections on Three Successive Days	Four Rabbits Receiving Two Injections, the Second Injection being Given on the Fifth Day
0.005	0.0012	0.001	0.00022
			

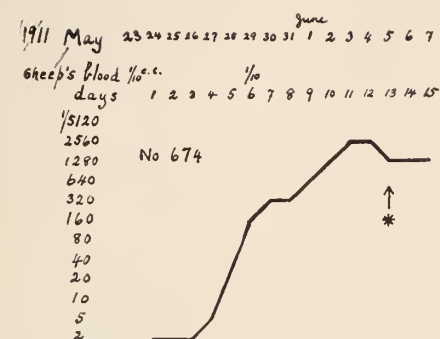
of 0.1 c.c. of washed sheep corpuscles at intervals of five, six, and seven days.

Following the first injection, the hemolysin content of the blood began to rise in all the animals after an interval of four or five days and increased daily up to the seventh day. Then, with one exception—Rabbit 684—the hemolytic strength remained unchanged for two days (Rabbits 674 and 643) or for three days (Rabbits 647, 642, and 644). This level marks the height of the response to the first injection. The effect of the second injection began, in some instances (Rabbits 674 and 642), to be apparent after three days, and in no animal was there any increase in the hemolysin content of the blood after the fifth day. The exceptional course of the curve in Rabbit 684, in which there was no pause in the increase of the rate of hemolysin production up to the final maximum, is due perhaps to two factors: first, the response to the second injection may have begun earlier than in the other rabbits; and secondly, the hemolysin content of the blood in this animal on the seventh day after the first injection was so low—1:40—that a relatively small increase could be easily detected.

The curve of hemolysin production as determined in this study differs in two respects from that of precipitin production as found by von Dungern. (a) In one instance, his rabbit "A," the height of the precipitin production was not reached until the eighth day after the first injection of the Majaplasma, and in the same animal the height of precipitin production following the second injection was not reached until the sixth day. (b) After reaching its height on the seventh or eighth day, the curve of precipitin production rapidly descends to a point at which it remains for a time stationary, whereas the curve of hemolysin production remains stationary from the day on which the height is reached.

The results of the preceding study may be applied as follows to the practical production in rabbits of a hemolytic serum against sheep corpuscles.

Two intravenous injections of 1, or at most 2 c.c. each of washed sheep corpuscles should be given at an interval of not less than four days to three rabbits. At the end of five days after the second injection, at least one of the rabbits will almost always yield a strongly hemolytic serum the hemolytic potency of which will be stable. If it is desired not to kill the animals, almost as much blood usually can be obtained by bleeding from the ear vein on two successive days (say



* on June 5th this animal's blood consisted of 9 parts of serum and 1 part of corpuscles. The animal died during the night of June 7th.

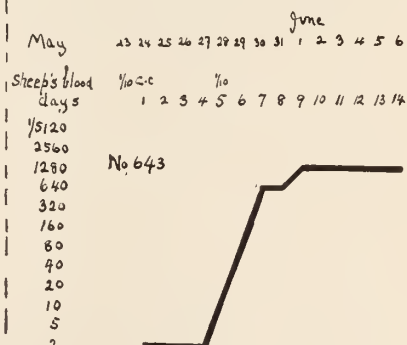
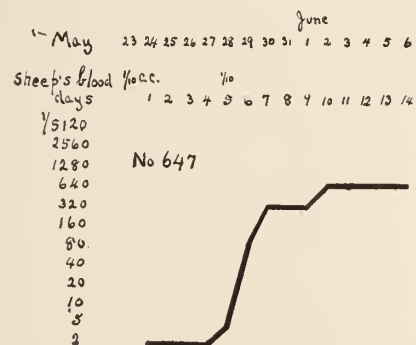
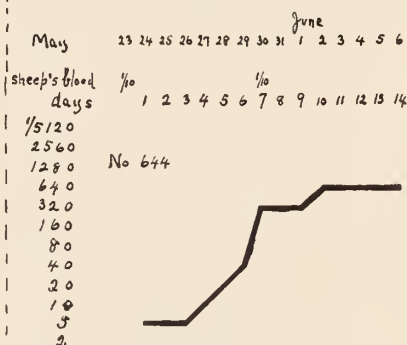
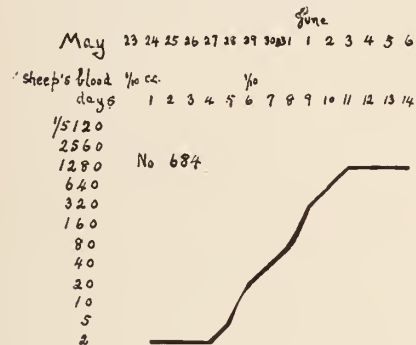
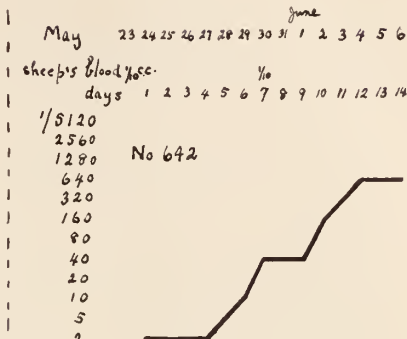


Chart 1.—The curve of hemolysin production in rabbits receiving two intravenous injections each of 0.1 c.c. of washed sheep corpuscles.

the sixth and seventh days after the second injection) as by bleeding to death. The potency of the serum obtained from the second bleeding is as high as that obtained from the first bleeding. The rabbits should not be used again for the production of amboceptor against sheep corpuscles.

In a single experiment in immunization with ox corpuscles, we have obtained results that are comparable with those obtained with sheep corpuscles.

Three normal rabbits received, at an interval of five days, two intravenous injections each of 1 c.c. of washed ox corpuscles. On the sixth day following the second injection, the hemolytic strength of the sera of the three rabbits was respectively 1:2500, 1:2500, and 1:320. After two months, during which the sera had been preserved in the ice-box with the addition of 0.25 percent of carbolic acid, the hemolytic strength of the sera was found to be unchanged.

THE THYROID OF THE GUINEA-PIG IN EXPERIMENTAL DIPHTHERIC INTOXICATION *

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INTRODUCTION

Rupert Farrant¹ has reported the experimental production of changes in the thyroid gland of the guinea-pig similar to those observed in the human subject in diphtheria. He states:

Firstly, the colloid becomes finely granular; secondly, it becomes vacuolated and partially absorbed; thirdly, an alteration occurs in the cells, they become more numerous, elongated, approaching the columnar, and arranged in masses; fourthly, the colloid becomes entirely absorbed and the walls of the vesicles become crenated and infolded; fifthly, the infolding and cell increase go on to transform the vesicles into solid masses of cells. These changes go on side by side so that several of them may be seen in the same section.

Farrant injected a number of guinea-pigs daily with the same dose of diphtheria toxin, which varied in strength from the minimal lethal dose to one-eighth part of it. Half the number of animals also were fed thyroid powder by mouth. These survived the animals not fed with this substance by one or two days. The doses of toxin were continued until the animals died, no mention being made of how soon after death the thyroids were removed to fixing fluids. He concludes that in the thyroid gland of the guinea-pig colloid absorption and cell reaction are produced by diphtheria toxin similar to the changes in the thyroid gland described in human diphtheria.

While working on thyroid problems, the writer was asked whether the changes described by Farrant could be produced in guinea-pigs by injections of diphtheria toxin given in such a way as to produce a course of toxemia similar to that in the human subject.

EXPERIMENTS

If we conceive that the initial toxin output in diphtheria in the human patient is not necessarily lethal, but that death is the consequence of a gradual overpowering of the defenses of the body, taking place in from one day or two, to a week or more, we should begin

* Received for publication June 12, 1915.

1. Proc. Roy. Soc. Med., 1913, 6,¹ p. 21.

injections of toxin in doses considerably smaller than the lethal dose. With this in mind, sixteen guinea-pigs, varying in weight from 230 to 400 grams, were injected under aseptic precautions with an initial dose of 0.001 c.c. of a toxin, three weeks old, the minimal lethal dose of which was 0.007 c.c. The consecutive doses were varied in such a way as to simulate the course of diphtheric intoxication of varying severity (Table 1).

In order to avoid postmortem changes, the animals were watched closely, and as soon as they showed signs of oncoming death they were killed by bleeding from the heart, with the exception of Guinea-pigs 3, 5, 7, 8, and 12, which were killed at earlier periods. This was done with the possibility in mind that the repair of possible glandular changes might occur early in milder toxemias; that is, as soon as the system of internal secretions had adjusted itself to the circumstances. Therefore, animals were selected at random between the third and twelfth days of the course of injections in the hope of being able to demonstrate such lesions. In all cases a careful autopsy was made, the thyroids having been dissected out with all possible care so as to exclude traumatic changes. The thyroid itself at no time was handled, but was taken out with the surrounding connective tissue.

In a previous study of some thirty thyroids of apparently normal guinea-pigs, it was observed that the common fixing agents produce changes in the colloid which easily might lead to errors in the interpretation of colloid changes in an experimental study. To avoid this, one lobe of the thyroid was fixed for twenty-four hours in Zenker's fixing reagent without acetic acid, and the other in Bensley's osmic bichromate acetic acid mixture. The latter had given the best results in the preservation of colloid in the normal thyroids. After the fixed tissues had been washed in running water for several minutes, they were submitted to a very gradual dehydration in alcohols of increasing strength, twenty-four hours being allowed for each increase. The time for absolute alcohol was cut down to a few hours, as the latter tends to shrink the colloid. The tissues, after being passed through bergamot oil, bergamot oil-paraffin, and paraffin in the usual way, were embedded in 62 C. paraffin. Serial sections from 3 to 5 microns thick were stained by the neutral gentian method, safranin acid violet and fuchsin-methyl-green methods, as described by Bensley,² with Mallory's anilin blue connective tissue stain, and with hematoxylin and eosin.

TABLE 1
DAYS AND DOSES OF INJECTIONS

[illegible]

In the thyroids of the normal guinea-pigs, fixed and stained in this way, it was found that vacuolation of the colloid is a common occurrence and is due to the fixing agents, as it is not seen in the fresh, unstained tissue. It was found further that the density of the colloid, as indicated by the staining intensity, varies to about the same degree and in the same proportion in the individual glands compared; also, that the size of the vesicles varies greatly in each individual gland, but that the compound glandular picture is always about the same in different guinea-pigs. As a general rule, the vesicles farthest away from the center of the gland are largest; but small vesicles are present also in this region. In the outer zone of vesicles the colloid appears to be more brittle, as indicated by the cracks made by the microtome knife.

It has occurred to me that the rather sudden and intense fixation of the outer vesicles leads to this phenomenon. The penetration of the fixing agents toward the more central vesicles is much slower and surely not as complete, and consequently the colloid preserves some of its flexibility.

"Langendorff-cells" are always found in the largest, commonly in the outer, vesicles, but they are present also in the central vesicles, only in less number.

Solid masses of cells, similar in structure to the cells of the vesicles (I shall call them "cell-buds" for short), are present in every normal gland, but they are scarce. At no time were the cells of these small masses found to undergo retrogressive changes. But neither were they found to be in an active state of hyperplasia, as indicated by the absence of mitotic figures, nor was there seen in these cells any colloid or its antecedent, as granules, which might point to a colloid secretion. The interpretation of these "cell-buds" is an open question. There are seen other very similar masses of cells which, to the unwary investigator who does not study serial sections, seem to be solid masses of cells. By following these through a series of sections, one learns that they are the top layers, so to speak, of vesicles. As the size of the individual vesicles varies greatly, it is easily understood that one may find many such "pseudo-cell-buds" at one level and few at another. Unless one makes a study of serial sections, this point will be overlooked, and if the mind of the investigator is in any way prejudiced in favor of hyperplasia of the gland a number of such "buds" in single sections will be interpreted wrongly.

Colloid was never found in the lymph spaces. With conventional stains, it is easy to mistake coagulated albuminous materials or densely

agglutinated red blood corpuscles for colloid, as they stain very much the same as do the latter, especially when overstained. It is needless to say that a careful study of the section with a good apochromatic oil-immersion lens will eliminate this error. Of great help in this respect are the different stains mentioned. Mallory's anilin blue stain for connective tissue has given excellent results in differentiating colloid. It brings out the colloid in an intense greenish blue, while the albuminous materials stain faintly sky-blue, the white fibrous tissue dark blue, and the red corpuscles red in Zenker's fixation without acetic acid. Also, the acetic acid osmic bichromate mixture gives good results with this stain, except that the colors are not as brilliantly differentiated.

In the series of normal thyroids, invagination (infolding) of the walls of a few vesicles was seen in two glands. In all the others it was completely absent.

On the basis of these results the study was undertaken of the thyroids of the animals given diphtheria toxin.

In no instance was there found any demonstrable change in the colloid. In the thyroid of Guinea-pig 8 there were a few vesicles filled with an irregular coagulum instead of colloid. Vacuolation of the colloid was not increased as compared with that of the normal glands. Neither was there any visible tendency of the walls of the vesicles to break down, crenate, or infold. The solid masses of cells, or solid "cell buds," were in proportion to the normal. Mitoses were not found anywhere. An increase in the number of parenchyma-cells or their general changing to the elongated type, which to some extent is present also in the normal gland, could not be demonstrated. Instead of this, it was observed that the cells of Guinea-pigs 11 and 12 were larger and clearer than normal and that they seemed to bulge considerably toward their respective glandular lumina.

In Guinea-pigs 4 and 8, many vesicles presented one cell in the individual row which was about four times as large as the normal. The cytoplasm of these cells was very clear, the reticulum very fine, and the nucleus about twice the size of the normal one. These cells were usually flanked by one or two Langendorff-cells. Whether these gland cells are in a state of active secretion could not be proved, as no secretion granules could be demonstrated.

In Guinea-pigs 6, 11, 14, 15, and 16, the number of Langendorff-cells was increased over the normal. Along with this a marked edema and a slight round-cell infiltration were noticed. A slight edema

together with a marked round-cell infiltration was seen in Guinea-pigs 7, 12, and 13.

Guinea-pigs 1, 2, 3, and 9 were entirely unchanged and 5 and 10 presented a slight round-cell infiltration alone. No colloid was seen in the lymph spaces.

DISCUSSION

If the results of this experimental study are compared with those of Farrant, the negative outcome of the writer's work is rather striking. As far as this work is concerned, postmortem changes were avoided entirely by taking out the glands from the freshly killed animal. The failure to observe early lesions was excluded by examining glands at different stages of the experiment. Traumatic changes were avoided by careful handling of the tissues concerned. Fixation artefacts were excluded by studying first a number of normal glands, fresh and fixed.

It may be argued that Farrant used larger doses early and repeated them oftener, and consequently obtained results which correspond in degree and kind to those found in severe human diphtheria. But he found such changes also when he used repeatedly small doses. In repeating his injections oftener, he did not allow the defense of the body to go into action. Undoubtedly, some such reaction occurs in human diphtheria. And as to the human material, we practically are confined to that from the severest and fatal toxemias. The changes in mild diphtheria have not been studied on account of lack of material.

The edema and round cell infiltration observed in those animals which were killed when dying seem to point to a vital reaction of the thyroid. But in most of these animals gross changes due to toxemia were seen in all the other organs also. Thus, the only sign of increased activity was perhaps the large, clear cells described. But, as they were found in only two glands of animals which had been given comparatively small doses, I do not think that we have the right to draw positive conclusions from this alone. Thus, the increase in Langendorff-cells in five glands is the only indication that there is some slight increase in thyroid reaction.

CONCLUSION

Experimental diphtheric toxemia produced in guinea-pigs in such a way as to simulate the course of human diphtheric toxemia does not lead to changes in the thyroid gland to the same degree as has been observed in the human thyroid gland in fatal diphtheria.

The changes observed in the thyroid of the guinea-pig are not sufficiently marked nor so uniformly parenchymatous as to allow us to conclude that this gland has a detoxicating action in experimental diphtheric toxemia.

LEPROSY—THE PRESENCE OF ACID-FAST BACILLI IN THE CIRCULATING BLOOD AND EXCRETIONS

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In the literature and text books on leprosy, there is much divergence of opinion regarding the presence of the acid-fast bacillus of leprosy in the blood and excretions of patients. The present paper is the result of systematic search in an attempt to analyze the cases occurring at the Penikese Leper Colony and to determine whether the acid-fast bacillus can or cannot be found in the blood, urine, feces, sputum, nasal mucus, and other excretions and discharges, so as to eliminate possible errors in research along other lines now being carried out.

REVIEW

The acid-fast bacillus of leprosy was originally discovered and described by Hansen in 1873-74, and later by Neisser, in the tissues and scrapings from lesions of leprosy. Since then, much attention has been paid to the manner in which the disease is communicated from one person to another, and to the site of the original lesion. In the last few years, the part insects play in spreading the disease has received considerable notice.

Nasal lesions with reference to the presence of the leprosy bacillus.—Ziemssen states that among the parts most affected are the nasal and oral mucous membranes and that even in the early stages ulcerations covered with corresponding crusts are met with, especially in the nose. Tilbury Fox in nearly the same terms calls attention to the involvement of the nasal mucous membrane. Arning and also Danielssen (quoted by Uhlenhuth and Steffenhagen) and Boeck state that leprosy ulcerations of the nose occur in anesthetic cases which otherwise present no ulcerations, and Arning in two cases found numerous bacilli in the discharge from nasal sores. Besnier states that the nasal mucus teems with bacilli. Leloir, however, notes that if the surfaces are infected, then the bacilli will be found in the nasal mucus. Goldschmidt advocates the examination of the nasal mucous membrane and nasal mucus. Falkao points out the fact of the prevalence of lesions in the nose and the frequent presence of the leprosy bacillus. Bibb occasionally found the bacillus in the secretions from the nose. Koch and Sticker state that the primary affection is in the nose, Koch basing his opinion on his experience of examining some 400 patients in India and Egypt. Kolle also directs attention to the condition of the nose and the nasopharyngeal passages in cases of leprosy. Gluck—altho not specifically examining nasal secretions—describes in great detail the pathologic changes and the presence of the bacillus there. Jeanselme and Laurence consider that the bacilli-

laden nasal mucus plays a rôle in the propagation of the disease. Fraenkel presents the case of a man who had suffered for three years from epistaxis and in whom he had found the bacillus in the nasal mucus. Thiroux found the bacillus in 39 percent of his cases. Black states that almost all mixed and nodular cases suffer from rhinitis and discharge bacilli with the nasal secretion. Thomson reports one case in which he found the typical bacillus of leprosy in the smear of the nasal mucus. Brinckerhoff and Moore lay importance on the examination of the nasal secretions for the detection of incipient cases of leprosy. Their positive results demonstrate the value of this means of bacteriologic diagnosis. Kitasato reports the presence of bacilli in 50 to 60 percent of the secretions of patients. Marchoux, after treating the patient with potassium iodid to produce a rhinitis, discovered in the nasal mucus the bacillus of leprosy. Leboeuf found the bacillus in the nasal mucus in 159 of 224 cases, the greatest percentage being among the tubercular patients. Jeanselme states that the nasal symptoms are commonly the first indication of the disease and thinks the rhinitis of leprosy one of the most prominent agents in the dissemination of the disease. Joyeux also reports the finding of bacilli in nasal mucus during attacks of coryza. Manson states that the bacillus is abundant in the purulent discharges from the nose. He indicates here that ulceration of the nasal tissues must have occurred. Hollmann presents an excellent article on the results of 329 examinations of the secretions of lepers, demonstrating the presence of the leprosy bacillus in 89.6 percent of nodular cases, 45.4 percent of anesthetic, and 66.6 percent of mixed cases. Thibault, in thirty cases examined, found the bacillus in the nasal mucus of twenty, potassium iodid having been used to provoke secretions. Leredde and Pautrier, and Pautrier report positive results in four cases after having given potassium iodid to induce rhinitis. Hollmann, as the result of the examination of the nasal mucous membrane of 500 lepers, found that there were lesions present in 410 cases.

While not all investigators are of the opinion that the leprosy bacillus can be detected in the nasal mucus, the majority believe that the earliest lesion is found in the nasal mucous membrane; others are either conservative on this last point or are positive in declaring that the nose is not the site of the primary lesion. A few assert that the bacillus is rarely or never found in the nasal secretions, especially in anesthetic cases.

Morrow states that the bacilli are absent from all physiologic secretions. McDonald, with regard to the bacillus in physiologic secretions, makes this statement: "After many faithful trials in every stage of the disease, I have found them so seldom that I now attach but little importance to it as a means of diagnosis." Black and Schilling state that the bacilli are not found in the nasal secretions of anesthetic or macular cases. Turner writes, "Never yet have I seen an ulcer in the nasal passages appear as a first indication of leprosy; it is rarely an early lesion."

The following paragraph from Mercier's article on Leper Houses and Mediaeval Hospitals, wherein he quotes from John Trevisa's translation from the Latin of Bartholemy Glanville, may be of interest. "Redde Whelkes and

Pymples in the face — out of whom oftenne runne Blood and Matter; in such the nose swollen and ben grete, the vertue of smellynge fayleth and the Brethe stynkyth right fowle."

Sputum.—As is well known, the mouth, pharynx, larynx, and vocal cords are commonly affected in leprosy; the bronchi and lungs relatively seldom. Therefore, the fact that bacilli are found may indicate either that the bacilli are most likely from the open lesions located in different parts of the respiratory tract, or that tuberculosis of the lungs is superimposed on leprosy. Unless investigators can exclude by animal inoculation the possibility that tuberculosis is present, the results obtained by examining sputum are of little importance.

The following are the only references on this phase of the subject that I have been able to find.

Besnier notes that the sputum usually contains numerous bacilli. Bibb found the bacillus occasionally in the sputum. Sticker reported that the sputum of 83 percent of his cases was positive. Spiegel found it swarming with bacilli in cigar-like masses. Uhlenhuth and Steffenhagen, by using antiformin, demonstrated the presence of the bacilli in sputum. Hollmann found it positive in only one case out of a total of thirty-one. Rosenau states that the bacillus does not occur in the expectoration from the lungs.

Saliva.—To a large extent the same is true of examination of the saliva. Consequently, unless the methods used by investigators are given, the results are of little value. Leloir states that he found the bacillus of leprosy in the saliva, but modifies his statement by saying that the surfaces must be infected. Morrow affirms that the bacillus is not found in the saliva, but Hollmann found the saliva to contain the bacillus in 21.7 percent of his cases of leprosy, all of the nodular type.

Urine.—The failure of investigators to state their methods in obtaining the urine and the fact that the exterior of the genitals is liable to be soiled and is often found to be the site of open lesions, makes the value of their results questionable. Interest is increased by the fact, quoted by Arning, that the Chinese believed the urine responsible for the spread of the disease. Many authors state that the bacillus is found in the urine but only during febrile exacerbations of the disease, and others that the bacillus can be induced by drugs to appear in the urine.

Arning, in his report to the Board of Health of Hawaii in 1886, Besnier and also Leloir, Morrow, McNutt, Bibb and McLeod — all state that the bacillus cannot be found in the urine. Since their work was done, however, there have been many statements to the contrary. The positive results may be entirely due to an improvement in technic. Lagane in 1912 and 1913 reports finding the bacillus in four of five cases. He states, however, that the bacilluria is

irregular, the bacilli probably appearing only during an acute attack. In three of his cases, the bacilli appeared after the giving of arsenobenzol. Rosenau believes that they are only occasionally found. DeBeurmann and Gougerot found the bacilli in the urine. Hollmann found it in 7.1 percent of his cases, eight being nodular leprosy. Stitt states that the "bacilli may be eliminated by the urine during occasions of fever."

Blood.—The presence of the bacillus in the blood is a more important question and has some bearing on the problem of transmission of the disease through the bite of insects. The technic is also important.

Ziemssen (1885) states that he had never succeeded in a single instance in demonstrating bacilli in the lumen of blood vessels, or with dry preparations of blood. He notes, however, that if blood is drawn from leprosy lesions, bacilli get into the escaping blood mechanically. Arning also states that if the blood is taken with all due precaution it does not contain the bacillus. Leloir after many preparations from five cases found the bacillus in one case. He thinks that the blood does not contain the bacillus permanently and that if it is introduced into the blood, it disappears rapidly. Besnier states that the bacillus is absent.

Hyde states that "their occurrence in the blood, an essential point in the demonstration of their etiologic importance, has been recognized solely by Hillairet and Gaucher." Neisser also declares that he never found the bacilli in the circulating blood. Müller, Köbner (quoted by Wolters), and also Majocchi and Pellizari (quoted by Ziemann) found the bacilli in the blood during the eruptive period. Jaja always found the bacilli in the blood, but his methods of skin puncture probably account for this result. Cornil and Babes found the bacilli in the blood by puncturing glands. (Here evidently the bacilli were from the gland tissue and not from the circulating blood.) Morrow states that the bacillus is not found in the blood. McNutt asserts that it cannot be found in the blood, not even during the febrile attacks. Bibb and Klingmuller found the bacilli in the blood. Gravagna-Catania's statement is of importance. In two of his cases, which were supposed to be stationary cases, the bacilli were found in the blood. Deycke-Pascha, performing an autopsy on a case of leprosy in a Jew, found that the blood contained many leprosy bacilli. Marchoux found them in the blood and not during the fever period. He also quotes Wolters, Wolff, and Doutrelepon as having found them in the blood. Ohashi found the blood positive in 80 percent of nodular, and 20 percent of anesthetic cases. Sugai and Monobe report six of ten cases positive; they also found the bacilli in the placenta of two of five anesthetic cases, in two of seven nodular cases, in the blood of ten of twelve new-born children, and also in a seventeen-month-old child. Crow reports the finding of the bacilli in twenty-one of twenty-four cases, of different types of the disease, mostly nodular. Manson states that they occasionally occur free in the blood or in leukocytes; he also states that if tuberculin is given it causes the appearance of bacilli in the blood. Lagane, in examining three cases of different types, did not, on first examination, find the bacillus, but, upon giving arsenobenzol, found it in both urine and blood in two cases. Kalindero found it in one case. In 1913 we have a

long series of favorable reports on the presence of the bacilli in the blood. DeBeurmann and Gougerot, Rabinowitsch and Reenstierna give positive, altho limited, reports. Thibault examined the blood of thirty patients and found the bacilli in seven. Stitt sums up the results by stating that the "leprosy bacilli are apt to be found in the blood of nodular cases, especially at the time of the febrile accessions," and that the "bacilli are present in the blood of cases of nodular leprosy rather constantly but less so in that of cases of nerve leprosy." Turner states that "the bacillus of leprosy may be met with wherever the blood circulates. To see them clinging to the endothelium of the capillaries causes one to wonder that they are not more common in the circulation."

Numerous statements appear regarding the finding of the lumen of blood vessels filled with bacilli, the presence of thrombi and occlusion of the vessels. Gluck, Danielssen, and Boeck all give details of the changes found in the blood vessels, the infiltration and thickening of the adventitia, small cell infiltration of the muscularis, and a high grade thickening of the intima, with numerous masses of bacilli scattered throughout, the infiltration of the same, and new formation of vessels. These changes all point to the reason for the presence of bacilli in the blood stream.

Feces.—The consensus of opinion is that ulceration of the intestine is necessary to the bacillus' being found in the intestinal discharges. It is also likely that material from the mouth, nose, and throat containing large numbers of bacilli may be swallowed. It is probable also that the bacilli may be found apart from these two causes.

Arning thought that the feces might play a part in spreading the disease, especially in primitive communities. The bacilli will be found especially in diarrhea. In 1886, in his report to the Hawaiian Board of Health, he again mentions finding the bacilli in the feces. Bibb never found the bacilli in the feces. Boeck also lays stress on the danger of the feces spreading the disease, having found the feces positive in one of three cases. Galli-Valerio, by treating the feces with antiformin, found the bacilli in both of two cases. Manson is doubtful whether they occur in the intestinal tract. Merian found the bacilli in two of three nodular cases, potassium iodid having been given in one case. Hollmann reports negative results in four cases.

It is rather surprising to find that so few report on this point. One difficulty, however, is recognized in the making of preparations from feces. The presence of an enormous number of organisms—some of them similar to the leprosy bacilli—necessitates the most careful search to obtain any result. It is wise therefore to be conservative in making any report of a positive nature.

Other secretions.—Regarding the presence of bacilli in other secretions, very few references are found. Leloir found the bacilli in the sperm, but not in the uterine or vaginal secretions. Klingmüller found it also in the sperm. Sugai and Monobe found it in the milk of two of twelve cases. English found it in the conjunctiva, and Hollmann found the bacilli in the sweat and tears in

14.2 percent of nodular cases. Besnier also reports finding them in the tears. Leloir states again that if the surfaces are infected the bacilli will be found in the tears.

METHODS

Feces.—Sterile porcelain jars were used for the collection of the specimens, from which a small amount, approximately one-fourth square inch, was taken and placed in 25 c.c. of 10 percent antiformin, thoroughly stirred, and allowed to stand for one hour; this was well shaken three or four times during the hour. One-half of the mixture was then transferred to a centrifuge tube and centrifugated for fifteen minutes, decanted, and the sediment washed in sterile water; once again it was centrifugated, decanted, and washed. After one more decanting, part of the sediment was spread fairly thick on a slide, fixed, and stained.

Sputum.—The usual method of collecting and making smears as for tuberculosis was used. Later it was found that using antiformin, washing with sterile water, and examining the sediment after centrifugation was the better method.

Nasal smears.—These were obtained directly from the free excretion (when obtainable). Smears were made, by means of a small, tightly woven cotton swab, directly on to clean slides. Tears were obtained in the same manner.

Urine.—In all cases during the last examination care was taken to have the external genitals clean, to discard the first part of the urine, and to see that the receptacles were sterile. Care was also taken that the hands of the patients should not come into contact with the urine or the receptacle. More care was exercised here than with any of the other specimens. The urine was examined on the same day as when passed and before examination was well shaken. All urine was centrifugated for thirty minutes. In a few cases, the sediment was treated with antiformin, washed with sterile water, and then the sediment examined; this method was used when the sediment was injected into guinea-pigs. Smears were made from the sediment after decanting the fluid. It was learned that if the bacilli were easily found in any given specimen it mattered very little how the sediment was obtained and treated. In all questionable cases, the urine sediment or the sputum was injected into guinea-pigs.

Blood.—This was obtained from the median vein at the bend of the arm because the skin here is seldom affected by the disease. By means of a sterile syringe, 5-10 c.c. of blood were taken and immediately emptied into a centrifuge tube containing a given hemolytic solution. Three methods of treating the blood were used, often two methods in the same case:

(a) By Crow's method take 5 c.c. of venous blood and put into a tube with 5 c.c. of a 2 percent sodium citrate in normal salt solution. Invert tube a few times, plug, place in cold place for sediment and next day take upper layer of sediment. Make thick smears, dry, place in distilled water five to fifteen minutes, then dry, fix and stain.

(b) By the Kurashigi-Schnitter method, take 1 c.c. of venous blood and put into a tube with 5 c.c. of a 3 percent acetic acid solution. Allow to stand one-half hour, centrifugate one-half hour, decant. Dissolve sediment in 5 c.c. of concentrated antiformin and when dissolved add 5 c.c. absolute alcohol, centrifugate one-half hour, decant, and wash sediment in distilled water, centrifugate again, and spread sediment on slides and stain.

(c) By Rabinowitsch's method, take 10 c.c. of venous blood and put into a tube with 10 c.c. distilled water with 1 percent sodium citrate (sapotoxin can also be added). Shake, centrifugate, and decant. Wash sediment in distilled water, decant, and then add 10 percent antiformin solution and let stand one hour at 37 C. Centrifugate, decant, and wash sediment in distilled water, decant, and spread sediment on slides and stain.

In some instances, modifications were made in these methods, but the Kurashigi-Schnitter method proved by far the most satisfactory. In the preparation of all specimens, two smears were made in each case and in some instances, more. All smears were fixed by heat and all staining was done with carbolfuchsin (Verhoeff's solution) as follows: the smear was steamed for thirty seconds, decolorized with 1 percent acid-alcohol for various lengths of time, according to the thickness of the smear, washed thoroughly, and counter-stained with Loeffler's methylene blue. In some instances, 95 percent alcohol was used as a decolorizing agent in case acid-alcohol was not sufficient in thick smears.

GENERAL RESULTS

Blood.—Of 28 examinations among 16 patients, there were 9 positive, 3 questionable, and 16 negative. This represents 8 patients with positive and 8 patients with negative results.

Urine.—Of 41 examinations made on 16 patients, 16 were positive in 9 patients.

Feces.—Of 27 examinations of 16 patients, 10 were positive in 6 patients.

Sputum.—Here the positive results were more frequent: Of 41 results recorded among 16 patients, 32 were positive and 9 negative, 14 patients giving positive and 2 patients negative sputum.

Nasal Mucus.—Sixteen patients with 41 examinations gave 34 positive, 6 negative, and 1 questionable result. In other words, 14 patients had bacilli in the nasal mucus and 2 did not.

Ulcerations.—Of 15 patients that had bullae, ulcers, and pustules, those having bullae gave 5 results positive and 10 negative; ulcers, 13 positive and 2 negative; and pustules, 7 positive and 8 negative.

Tears.—Negative in 26 examinations with 13 patients.

GENERAL DISCUSSION

In view of the criticism of previous papers in this respect, care was taken to obtain, prepare, and stain specimens as uniformly as possible. Every precaution was taken to eliminate outside bacteria; that is, by the use of sterile water, clean slides, etc. Examining the slides was an extremely arduous task, consuming an enormous amount of time and

patience. No slide was considered positive unless bacilli were found in several fields or, in the case of sputum or similar specimens, in groups. In examining the specimens, a large number of organisms were found, some acid-fast and some similar in many respects to the acid-fast bacillus of leprosy. The blood was not taken during febrile attacks. No acid-fast bacilli other than those agreeing with the type of Hansen were considered, and to avoid error of judgment, reference was frequently made to slides obtained from lesions of leprosy showing typical bacilli, singly and in groups. Apart from the three series of examinations, a great number of individual examinations have been made during the two years and notes kept of the results. Smears were made on the same day that specimens were obtained; in the case of urine, this precaution was obviously necessary. In the case of blood, urine, and fecal specimens, the possibility of the presence of local lesions was kept in mind. Lesions in the kidneys are extremely rare; intestinal ulceration is more common.

From the table representing examinations in June and December, 1914, and March, 1915, it will be seen that the results are uniform from the three series in only a few cases. In one case, an anesthetic, there was only one positive result. It may have been an error in technic that there were not more positive results. The nasal secretions and sputum gave the greatest number of positive and uniform results. The fact that the air passages are so early and seriously affected and constantly irritated and that bacilli from open lesions can so easily be taken up by the secretions, will account for the great number of positive findings. The four cases which gave the largest number of positive results were all ulcerative nodular cases, in a very advanced stage of the disease. The feces and the blood gave the least number of positive results. It is difficult to understand why the specimens of feces did not show better results, as, I believed, the sputum swallowed might also have added to the frequency of the occurrence of the bacilli. During diarrhea, the bacilli were more easily and more abundantly found. They were found however in cases which did not suffer from intestinal disturbances. The blood and urine should have given more uniform results, and, in cases where the bacilli were fairly readily found in the urine, a more eager attempt was made to find the bacilli in the blood specimens. The findings in the smears from ulcers, bullae, etc., need no further explanation.

TABLE 1
RESULTS OF THE EXAMINATION OF BLOOD, ETC., TO DETERMINE THE PRESENCE OF ACID-FAST BACILLI

Patient	Type of Disease	Stage	Blood			Urine			Sputum			Feces		Nasal Mucus		Tears		Bullae		Ulcers		Pustules	
			June 1914	March 1915		June 1914	Dec. 1914	March 1915	June 1914	Dec. 1914	March 1915	Dec. 1914	March 1915	June 1914	Dec. 1914	March 1915	March 1915	June 1914	June 1914	June 1914	June 1914	June 1914	June 1914
F. B.	Nodule ulcerative	Advanced.....	0	+	+	+	+	+	+	+	+	0	+	+	+	0	0	0	+	+	+	+	+
*H. B.	Mixed.....	Advanced.....	0	0	0	0	0	0	+	+	+	0	0	+	+	0	0	+	+	+	+	0	0
†M. C.	Nodular.....	Far advanced	0	+	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0
M. G.	Nodular.....	Advanced.....	0	+	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*L. D.	Mixed.....	Advanced.....	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*S. G.	Nodular.....	Advanced.....	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M. M.	Anesthetic..	Advanced.....	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M. P.	Nodular.....	Advanced.....	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
†L. Q.	Nodular.....	Advanced.....	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
W. Q.	Mixed.....	Early.....	+	?	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
*A. T.	Nodular.....	Advanced.....	+	?	0	0	0	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. T.	Nodular ulcerative	Advanced.....	0	?	0	+	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
†L. U.	Nodular ulcerative	Advanced.....	0	N.Ex.	0	0	0	+	+	+	+	0	0	+	+	+	0	0	+	+	+	+	+
H. K.	Nodular.....	Early.....	..	+	0	0	0	+	0	0	+	0	0	+	+	+	..	0	0	0	0	0	0
H. O.	Anesthetic..	Early.....	0	..	0	0	0	0	0	0	0	0
*F. P.	Nodular.....	Far advanced	0	..	0	0	0	0	0	0	0	0
J. N.	Nodular.....	Far advanced	0	..	0	0	0	0	0	0	0	0

* Epistaxis occurred in these cases.

† Diarrhea occurred in these cases.

Blood was not examined during febrile attacks.

It is evident, therefore, that the only question which materially interferes with the conclusions is the inability to identify absolutely the acid-fast bacillus as being the acid-fast bacillus of leprosy. The possibility of its being the tubercle bacillus can be ruled out by the failure to obtain positive results by animal inoculation or by cultivation. Inoculations of guinea-pigs with the sediment of urine and sputum resulted negatively. The numerous cultivation experiments for an acid-fast bacillus have also proved negative. To a certain extent, the possibility of an acid-fast bacillus from water or contamination is unlikely, on account of the care taken to avoid this possibility. The routine method of treating, staining, and examining specimens, the adherence to a type bacillus, and especially the fact that the bacilli occurred in groups lessens the chance of error that we are dealing with other than the acid-fast bacillus of leprosy. In many respects, the questions raised here are similar to those which have been discussed in tuberculosis.

CONCLUSIONS

Bacilli resembling the typical acid-fast bacilli of leprosy were found in the circulating blood and excretions examined, frequently enough to warrant the statement that the blood, urine, feces, sputum, nasal excretions, etc., contain the bacilli and consequently may play a part in the spread of the disease, especially where unhygienic conditions prevail and the disease is prevalent.

The occurrence of symptoms of toxic-febrile attacks, etc., are more easily understandable when the fact is accepted that the bacilli are found in the blood stream and excretions.

The presence of bacilli in the blood and in the various excreta as determined, warrants the assumption that insect transmission of leprosy is a possibility.

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THE SEROLOGIC DIAGNOSIS OF LEPROSY *

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It is our intention to answer, if possible, the following questions bearing on the serologic diagnosis of leprosy.

1. To what extent does the serum of a leper react in complement fixation with tuberculin, about which much has been written?

2. What relation does this phenomenon bear to the Wassermann reaction?

3. What attitude does tuberculin show toward non-leprous serum in regard to complement fixation?

4. Does or does not the leprous blood contain some antigens produced by the bacilli in the body? That is, may the blood contain such properties as have arisen from the bacilli themselves, or the products of the organic cells produced by the irritant effect of the bacilli?

5. Might these properties remain inactive in complement-fixation tests because of the immune properties produced by such antigens?

6. Will such properties be shown, as a result of comparative study, to be present in the blood of a patient suffering from diseases other than leprosy?

EXPERIMENTS

The following materials were employed in our experiments:

Antigen.—1. Koch's old tuberculin (prepared by the Imperial Institute for the Study of Infectious Diseases, Tokio). Each examination was made with 0.2 c.c. of the dilution in the proportions of 1:20, 1:40, and sometimes 1:10.

2. Alcoholic extract of the heart of a guinea-pig, diluted with physiologic salt solution. The heart was washed in physiologic salt solution and cut up until it presented a pasty appearance, to which twenty volumes of 90 percent alcohol were added. This was allowed to stand for two or more days at room temperature, being shaken at intervals only. Before use it was evaporated at 37 C. The remaining sticky mass was made into a suspension with six volumes of 0.85 percent sterile salt solution, to which carbohc acid in the proportion of 0.5 percent was added. It was then thoroughly shaken and a slightly whitish solution obtained. Of this original solution, 0.4 c.c. was used as the standard quantity, which was diluted sufficiently to avoid auto-inhibition. For the sake of convenience, this may be called Wassermann's syphilis antigen.

3. Leprous nodule. Leprous cutaneous nodules were taken out, the adherent connective and fatty tissue removed, and the remainder washed in physiologic salt solution and cut up until reduced to a pasty mass. It was then treated in the same manner as the guinea-pig heart.

* Received for publication June 18, 1915.

4. Clot of blood. (a) A clot of leprous blood, which was separated from a serum that had given a positive reaction with leprous antigen, and which remained quite free from the wall of the sterile tube containing the blood while the separation of the serum and clot was taking place, was removed after complete separation of the serum had been effected by standing it for twenty-four to forty-eight hours at room temperature or for two hours in an incubator. The clot was then washed in 0.85 percent salt solution, and cut up into a pasty mass. It was then treated exactly like the heart extract. For the sake of convenience, this may be termed the "leprous positive clot antigen."

(b) A clot of blood, the serum of which was negative in the complement-fixation test with leprous antigen, was treated in the same manner as the clot in (a) and the product called the "leprous negative clot antigen."

(c) A clot of blood from a non-leprous patient (i. e., syphilitic) which had given negative results in the complement-fixation test with the leprous nodule antigen, was treated in the manner described in (a) and the product called the "non-leprous clot antigen."

(d) A clot of blood from a guinea-pig was treated in the same manner and the product called the "guinea-pig clot antigen."

Salt Solution.—A normal solution in the proportion of 0.85 percent was employed.

Complement.—Fresh serum from a normal guinea-pig.

Hemolytic Amboceptor.—Serum of a rabbit that had been immunized with goat corpuscles, and which was proved to have a hemolytic efficiency as high as 2,000 to 4,000, was made into a dilution having three times the strength of the minimal efficiency.

Corpuscles.—Goat corpuscles were centrifugated three times and washed with salt solution and then a suspension of twenty times the volume of the original blood was made with normal salt solution. These were treated according to the following method: The dilute solution of hemolytic amboceptor and the corpuscle suspension were mixed in equal parts, and after thorough stirring the mixture was kept for thirty minutes at 37 C., when it was ready for use as a hemolytic system. One cubic centimeter was used for each test.

The test may be tabulated as follows:

TABLE 1
COMPLEMENT-FIXATION TEST

Test Tubes	Diluted Serum of Patient	Antigen	Ten Parts Dilution of Complement	Additional Salt Solution	Hemolytic System
Control 1	0.5 (0.2)	...	0.5	0.5	1.0
Experiment 1 ...	0.5 (0.1)	0.2	0.5	0.3	1.0
Experiment 2 ...	0.5 (0.05)	0.2	0.5	0.3	1.0
Control 2	0.4	0.5	0.6	1.0
Control 3	0.5	1.0	0.5
Control 4	1.5	1.0

The figures in parentheses in the column of patient's blood serum show the total amounts of the serum.

All the preparations were mixed and incubated together for one hour at 37 C. before adding the hemolytic system. After the addition of the hemolytic system, they were kept at 37 C. for one hour, after which they were shaken thoroughly and then again left for one hour and the results recorded.

Antigens and Degrees of Reactions

Patients	Old Tuberculin		Wasser- mann's Syphilis Antigen		Leprous Positive Clot Antigen				Leprous Negative Clot Antigen		Non-leprous Clot Antigen		Guinea-pig Clot Antigen	
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1. M. Severe tubercular leprosy	++	++	+++++	+++++	++	++	—	—	+++++	+++++
2. F. Severe tubercular leprosy	+++	+++	+++++	+++++	+++++	+++++	+++	+++	++	++	—	—
3. M. Severe anesthetic leprosy	+++	+++	+++++	+++++	+++++	+++++	+++	+++	++	++	—	—
4. M. Light anesthetic leprosy	++	++	—	—	—	—	—	—
5. F. Severe anesthetic leprosy	+	+	—	—	—	—	—	—
6. M. Light tubercular leprosy	—	—	—	—
7. M. Light tubercular leprosy	—	—	—	—
8. M. Mild anesthetic leprosy	++	++	+++	+++	+++++	+++++
9. F. Mild tubercular leprosy	++	++	++	++	+++++	+++++
10. M. Light anesthetic leprosy	—	—	—	—	—	—
11. M. Severe anesthetic leprosy	++	++	+++	+++
12. M. Severe tubercular leprosy	++	++	+++	+++
13. M. Light anesthetic leprosy	+	+	+++	+++
14. M. Mild tubercular leprosy	+	+	++	++
15. M. Light anesthetic leprosy	+	+	++	++
16. M. Light tubercular leprosy	—	—	++	++
17. M. Mild anesthetic leprosy	—	—	++	++
18. M. Light tubercular leprosy	++	++	+++	+++
19. M. Severe tubercular leprosy	+++	+++	+++	+++
20. M. Severe tubercular leprosy	+++	+++	+++	+++
21. M. Mild tubercular leprosy	+++	+++	+++	+++
22. M. Mild tubercular leprosy	+++	+++	+++	+++
23. M. Severe tubercular leprosy	++	++	+++	+++
24. M. Severe anesthetic leprosy	+	+	++	++
25. M. Light tubercular leprosy	+++	+++	+++	+++
26. M. Severe tubercular leprosy	+++	+++	+++	+++
27. F. Mild tubercular leprosy	++	++	++	++
28. M. Severe tubercular leprosy	+	+	++	++
29. F. Severe tubercular leprosy	+++	+++	+++	+++
30. F. Severe tubercular leprosy	+++	+++	+++	+++
31. M. Light anesthetic leprosy	+	+	++	++
32. M. Severe tubercular leprosy	+++	+++	+++	+++
33. F. Severe tubercular leprosy	+++	+++	+++	+++
34. F. Mild tubercular leprosy	+	+	++	++
35. M. Severe tubercular leprosy	+++	+++	+++	+++
36. M. Severe tubercular leprosy	++	++	++	++
37. M. Light tubercular leprosy	±	±	++	++
38. M. Severe tubercular leprosy	+++	+++	+++	+++
39. F. Light anesthetic leprosy	++	++	++	++
40. F. Light anesthetic leprosy	±	±	++	++
41. M. Severe anesthetic leprosy	++	++
42. M. Light tubercular leprosy	++	++
43. M. Light anesthetic leprosy	++	++
44. M. Light anesthetic leprosy	++	++
45. M. Light tubercular leprosy	++	++
46. M. Mild tubercular leprosy	++	++
47. M. Syphilis (?)	—	—	+++	+++
48. M. Syphilis	—	—	+++	+++
49. M. Syphilis	—	—	+++	+++
50. M. Syphilis	—	—	+++	+++
51. M. Syphilis (?)	±	±	+++	+++
52. M. Gonorrheal epididymitis	—	—	+++	+++
53. M. Tubercular arthritis (?)	—	—	+++	+++
54. F. Syphilis	—	—	+++	+++

* The serum of this patient showed auto-inhibition.

By severe nodular leprosy is meant patients having numerous nodules in the face and other parts of the body; by mild, those who have lesser nodules in the body; by light, those who have only regional affection with but a few nodules or a number of the old nodules being absorbed, the illness being in a state of suspension for the present. The degree of severity is determined either by the degree of deformity and emaciation in limbs, accompanied by difficulty of motion, or by cutaneous or muscular ulceration owing to the nutritive nervous system, or general appearance of the face.

The results of our experiments are given in Table 2. From Table 2 we shall summarize the effects produced by each antigen, separately, and make a critical study of the behavior of the serum.

Thirty-nine of the forty-two leprous sera examined gave unmistakable reactions (either positive or negative) with old tuberculin, 2 gave indistinct reactions, and 1 gave auto-inhibition. Positive and negative reactions are classified in Table 3 according to the type and the severity of the disease, in Table 4 according to severity only, and in Table 5 according to types.

TABLE 3
REACTIONS OF LEPROUS SERA WITH OLD TUBERCULIN

Cases	Number of Tests	Positive Results	Percentage of Positive Results
Severe tubercular leprosy.....	14	14	100.00
Mild tubercular leprosy.....	6	5	83.33
Light tubercular leprosy.....	4	2	50.00
Severe anesthetic leprosy.....	5	3	60.00
Mild anesthetic leprosy.....	3	0	0
Light anesthetic leprosy.....	7	3	42.85
Total	39	27	69.23

TABLE 4
REACTIONS OF LEPROUS SERA WITH OLD TUBERCULIN CLASSIFIED ACCORDING TO SEVERITY OF SYMPTOMS

Severity	Number of Cases	Positive Cases	Percentage of Positive Cases
Severe	19	17	89.48
Mild	9	5	55.56
Light	11	5	45.45

TABLE 5
REACTIONS OF LEPROUS SERA WITH OLD TUBERCULIN CLASSIFIED ACCORDING TO TYPES OF DISEASE

Type	Number of Cases	Positive Cases	Percentage of Positive Cases
Tubercular leprosy	24	21	87.50
Nervous leprosy	15	6	40.00

From Table 3 it is seen that a regular proportion exists between the reaction and the severity of the disease, when it is tubercular, while the same seems to apply in part to anesthetic leprosy. The reversed proportion that seems to exist in the light and mild forms must remain unexplained until more extensive tests can be made. It may possibly

be due to the fact that latent non-anesthetic lesions existed in the internal organs of the body and that the patient showed only slight nervous disorder upon superficial examination. If the results of the test be classified according to the types of the disease, it is seen that the tubercular leprosy produced twice as many positive cases as the nervous leprosy, while the percentage increased regularly according as the severity of the disease was augmented.

Möller¹ recently reported results of complement fixation with leprous serum by the use as antigens of old tuberculin, new tuberculin, bovine tuberculin (prepared by Höchst), and the suspension of the pulverized human tubercle bacilli (laboratory preparation), according to the directions of Koch. An interesting comparison is made between his results and our own in Table 6.

TABLE 6
MÖLLER'S RESULTS IN COMPLEMENT-FIXATION WITH LEPROUS SERUM COMPARED WITH NAKAJO AND ASAKURA'S RESULTS

Facts	Möller's Results	Nakajo and Asakura's Results
Total number of tests.....	32	39
Positive reactions	25 (78.12%)	27 (69.23%)
Percentage positive		
(a) Tubercular leprosy	95-100	87.50
(b) Anesthetic leprosy	25	40.00
Relation	In direct proportion	In direct proportion

Our results seem to be inferior to Möller's in respect to the percentage positive of the test. However, this difference may be annihilated if only such of Möller's cases be given as produced a positive reaction with the culture fluid; that is, a total of 20 cases (60.25 percent). The singular reversal that occurred between the percentages of the tubercular and nervous cases may be explained by the difference of view regarding the diagnosis; for in the lighter forms these distinctions are often difficult to establish. The average percentage of both tubercular and nervous cases was 62.50 in Möller's case, while in ours it was slightly higher (63.75). The more the infection tends to be tubercular in nature, the more positive will be the results obtained.

Möller classified as positive such cases as brought about even a slight complement-fixation reaction, and we also classed the cases that showed a slight hemolytic inhibition among the positive cases.

In short, Möller's results and our own appear on the whole to agree with one another. The reactions go parallel with the severity of the symptoms and the quantity of micro-organisms in the patient's body;

1. Deutsch. med. Wchnschr., 1913, 39, p. 595.

for in tubercular leprosy a high ratio of positive reactions is obtained, while in nervous cases, in which only a few bacilli are found, which are regarded by various authors as degenerated forms of leprosy bacilli, the reaction test in question may be regarded as having a close relation to the very nature of the disease, altho the reaction is by no means specific with tuberculin. Our present test, however, may be compared in its clinical importance to Wassermann's test for syphilis made with the heart extract of a normal guinea-pig as antigen. With regard to the question suggested by Meyer, that is, whether tuberculin might not produce positive reactions not only in syphilis, but in other diseases, our test, altho made in but few cases, gave the results shown in Table 7.

TABLE 7
COMPLEMENT-FIXATION WITH OLD TUBERCULIN IN DISEASES OTHER THAN LEPROSY

Kinds of Disease	Positive Reaction	Negative Reaction	Para-reaction
Syphilis	—	4 cases	1 case
Tubercular arthritis	—	1 case	—
Gonorrheal epididymitis	—	1 case	—

Let us review the results obtained with Wassermann's syphilis test and the old tuberculin test of twenty-one leprous and eight non-leprous sera, respectively. (One leprous serum that gave auto-inhibition was excluded.)

From Table 8 we see that fourteen of twenty-one cases (two-thirds) gave positive results in both tests, while five of the remaining seven gave positive reactions with old tuberculin alone, and one of the remaining two gave a para-reaction with the former and a slight positive reaction with the latter. In short, our experiment shows that leprous sera give a much greater number of positive results in complement-fixation tests with old tuberculin than with Wassermann's syphilitic antigens; while with syphilitic sera just the reverse obtains. Thus, we may infer from the results of our experiments that the leprous sera contain properties that would give positive reactions to both antigens, and the syphilitic sera those that would give positive reactions to Wassermann's antigen only. The sera of the patient suffering from gonorrheal epididymitis did not react to either antigen.

We shall now give the results of our complement-fixation tests with leprous and non-leprous sera and the leprous nodule antigen and old tuberculin.

From Table 9 we may see that nine of seventeen cases produced positive results in both tests. Thirteen of seventeen cases gave homo-

TABLE 8
RESULTS OBTAINED WITH WASSERMANN'S SYPHILIS TEST AND THE OLD TUBERCULIN TEST

Kinds of Disease	Total Tests	Wassermann's Reaction			Complement-Fixation vs. Old Tuberculin			Number of Reactions Agreeing in Either Test
		Positive	Negative	Para-reaction	Positive	Negative	Para-reaction	
Severe tubercular leprosy.....	11	10	1	..	11	10
Mild tubercular leprosy.....	4	1	3	..	4	1
Light tubercular leprosy.....	2	1	1	..	1	..	1	..
Severe anesthetic leprosy.....	2	2	2	2
Mild anesthetic leprosy.....
Light anesthetic leprosy.....	2	1	1	..	1	..	1	1
	21	15	6	..	19	..	2	14
Syphilis								
Syphilis(?)	4	4	4
Tubercular arthritis(?)	2	2	1
Gonorrheal epididymitis	1	1	1
	8	7	1	7

The two cases of suspected syphilis and the one case of suspected tuberculous (arthritis) were reported to be syphilis, while the gonorrheal epididymitis referred to us by Dr. Sugli, Chief of the Aomori Hospital, gave a negative Wassermann. These results agree with those obtained by us.

The two cases of light tubercular leprosy each alternately produced one instance of positive reaction; no coincident cases therefore occurred.

TABLE 9
COMPLEMENT-FIXATION TEST WITH LEPROUS AND NON-LEPROUS SERA AND THE LEPROUS NODULE ANTIGEN AND OLD TUBERCULIN

Kinds of Disease	Total Number of Tests	Complement-Fixation vs. Old Tuberculin			Complement-Fixation vs. Leprous Nodule Antigen			Number of Cases Agreeing	Number of Cases Not Agreeing
		Positive	Negative	Para-reaction	Positive	Negative	Para-reaction		
Severe tubercular leprosy.....	5	5	5	5	..
Mild tubercular leprosy.....	1	..	1	..	1	1
Light tubercular leprosy.....	2	1	1	..	1	1	..	2	..
Severe anesthetic leprosy.....	2	1	1	2	..	1	1
Mild anesthetic leprosy.....	2	..	2	2	..	2	..
Light anesthetic leprosy.....	5	2	3	..	2	3	..	3	2
	17	9	8	..	9	8	..	13	4
Syphilis	5	..	4	..	2	3	..	3	2

geneous results in both tests, seven of which were positive. Two of the remaining four cases that disagreed in both tests gave positive results with old tuberculin only, while the other two were positive with the leprous nodule antigen only. These positive reactions thus may be considered as being leprous in origin, for, as we saw in the second table, the leprous nodule antigen gave a strong positive reaction with syphilitic sera in two cases of five. Here, we wish to emphasize the importance of guarding against the production of a non-syphilitic positive reaction by testing the antigen with a number of non-syphilitic sera, for various acquired properties may be present in the organs that are to be used as antigens.

Let us now consider what is the relation existing between the results of the complement-fixation test of the leprous and of the non-leprous sera and the leprous positive clot antigen and old tuberculin.

TABLE 10

COMPLEMENT-FIXATION TEST WITH LEPROUS AND NON-LEPROUS SERA AND THE LEPROUS POSITIVE CLOT ANTIGEN AND OLD TUBERCULIN

Cases	Old Tuberculin		Positive Leprous Clot Antigen	
	Test-Tube 1	Test-Tube 2	Test-Tube 1	Test-Tube 2
1. M. Severe tubercular leprosy	+++	++	++++	++++
2. F. Severe tubercular leprosy	++	++	+++++	+++++
3. M. Severe anesthetic leprosy	++++	++++	+++++	+++++
4. F. Severe anesthetic leprosy	++	++	+	+
5. M. Light anesthetic leprosy	—	—	—	—
6. M. Light anesthetic tubercular leprosy	—	—	+	+
7. M. Mild anesthetic leprosy	—	—	—	—
8. M. Mild tubercular leprosy	++	+	+++++	+++++
9. F. Light anesthetic leprosy	—	—	—	—
10. Severe anesthetic leprosy	—	—	—	—
11. Mild anesthetic leprosy	—	—	—	—
12. M. Severe tubercular leprosy	+++++	+++++	+++++	+++++
13. M. Light nervous leprosy	+	+	+	+
14. M. Syphilis	—	—	—	—
15. M. Syphilis	—	—	—	—
16. M. Syphilis	—	—	—	—
17. M. Syphilis	—	—	—	—
18. M. Syphilis	±	±	+	+

From Table 10, it is seen that of thirteen cases, twelve gave homogeneous results in both tests, seven being positive and five negative. Of five cases of non-leprous patients (i. e., syphilitic), four agreed in both tests in being negative. There occurred no case showing non-conformity in the results. One instance of leprous serum that gave negative results with old tuberculin and positive with leprous positive clot antigen, and one of non-leprous serum that gave a para-reaction with old tuberculin and a slightly positive one with the leprous clot, constitute unimportant variations. These two materials, therefore, may be considered to have produced identical results. Moreover, strictly considered, the leprous positive clot showed some tendency to produce a non-leprous reaction because of the coinciding foreign properties that were found in the leprous nodule antigens.

Let us now consider the relation between the results obtained in the complement-fixation test with the leprous and non-leprous sera and the leprous negative clot antigen.

TABLE 11
COMPLEMENT-FIXATION WITH LEPROUS AND NON-LEPROUS SERA AND LEPROUS NEGATIVE CLOT ANTIGEN

Cases of Leprosy	Old Tuberculin		Leprous Negative Clot Antigen	
	Test-Tube 1	Test-Tube 2	Test-Tube 1	Test-Tube 2
1. F. Severe tubercular	++	++	—	—
2. M. Severe anesthetic	++++	++++	+	+
3. F. Severe anesthetic	++	++	—	—
4. M. Light anesthetic	—	—	—	—
5. M. Light anesthetic	+	+	—	—
6. M. Mild tubercular	—	—	—	—
7. M. Light tubercular	+++	+++	—	—
8. M. Mild anesthetic	—	—	—	—
9. M. Mild tubercular	—	—	—	—
10. M. Severe anesthetic	—	—	—	—

From Table 11, we see that only one case of the ten gave a slightly positive reaction with the leprous negative clot, all the other nine being negative, while five cases gave positive results with old tuberculin and no contradictory case occurred. Therefore, we may infer that the leprous negative clot contains little or no property that produces any reaction in the test in question; and that the leprous negative clot

produces no conforming results with old tuberculin in the leprous complement-fixation test different from those produced by the leprous positive clot.

We shall now give the results of the tests made with syphilitic blood clot and old tuberculin and leprous and non-leprous sera.

TABLE 12

COMPLEMENT-FIXATION TEST WITH LEPROUS AND NON-LEPROUS SERA, THE SYPHILITIC BLOOD CLOT AND OLD TUBERCULIN BEING USED AS ANTIGENS

Cases	Old Tuberculin		Non-Leprous Clot Antigen	
	Test-Tube 1	Test-Tube 2	Test-Tube 1	Test-Tube 2
1. M. Severe tubercular leprosy	+++	++	—	—
2. F. Severe tubercular leprosy	++	++	—	—
3. M. Severe anesthetic leprosy	++++	++++	—	—
4. F. Severe anesthetic leprosy	++	++	—	—
5. M. Light anesthetic leprosy	—	—	—	—
6. M. Light tubercular leprosy	—	—	—	—
7. M. Severe tubercular leprosy	++++	++++	—	—
8. M. Light tubercular leprosy	+++	+++	—	—
9. M. Mild anesthetic leprosy	—	—	—	—
10. M. Light tubercular leprosy	—	—	—	—
1. M. Syphilis	—	—	—	—
2. M. Syphilis	—	—	—	—
3. M. Syphilis	—	—	—	—
4. M. Syphilis	—	—	—	—
5. M. Syphilis	±	±	—	—
6. M. Gonorrheal epididymitis	—	—	—	—
7. M. Syphilis	—	—	—	—

From Table 12, we may conclude that non-leprous clot possesses no property like that of leprous positive clot, for the non-leprous clot antigen produced negative results with all the sera of ten lepers, as well as with that of seven non-leprous patients, while tuberculin reacted positively in six of the ten cases.

Finally, we shall compare the results obtained in the tests made with guinea-pig blood clot and old tuberculin on leprous and non-leprous sera.

TABLE 13

COMPLEMENT-FIXATION TEST WITH LEPROUS AND NON-LEPROUS SERA, GUINEA-PIG BLOOD CLOT
AND OLD TUBERCULIN BEING USED AS ANTIGENS

Cases	Old Tuberculin		Blood Clot of Guinea-Pig	
	Test-Tube 1	Test-Tube 2	Test-Tube 1	Test-Tube 2
1. M. Severe tubercular leprosy	+++	++	+++++	+++++
2. M. Mild anesthetic leprosy	—	—	—	—
3. M. Mild tubercular leprosy	++	+	+++++	+++++
4. F. Light anesthetic leprosy	—	—	—	—
5. M. Severe anesthetic leprosy	—	—	—	—
6. M. Syphilis	—	—	—	—

Tho our test was made on a small number of cases, the results in both instances agreed perfectly.

This report concerns the complement-fixation tests made upon the sera of forty-six lepers and eight non-leprous patients, the total number of tests being 155.

SUMMARY

Our results in complement-fixation tests with leprous sera and old tuberculin, considered from the standpoint of the type of the disease, show that a higher percentage positive, that is, 87.5 percent, was obtained in tubercular leprosy, than was obtained in nervous leprosy—40 percent. Classified according to the severity of the symptoms, these results are: 89.48 percent positive cases, severe; 55.56 percent, mild; and 45.45 percent, slight. From these facts, we may conclude that the reaction produced by old tuberculin bears a direct relation to the nature of leprosy, tho, as Babes⁴ and Möller¹ affirm, it is never specific. Moreover, the heart extract of a guinea-pig may be substituted for it in the same way as in the Wassermann² test.

As Meier³ has pointed out, if there is any doubt as to the disease (whether syphilis or leprosy) from which a patient is suffering, it will probably prove to be leprosy if the complement-fixation tests with both old tuberculin and Wassermann's² antigen give positive results. If the test reacts positively only with old tuberculin, the disease is unmistakably leprosy; but negative results are not sufficient to exclude the case as leprosy.

2. Hemolysine, Zytotoxine und Praezipitine, 1910, p. 79.

3. Verhandl. d. 2. Internat. Leprakonferenz in Bergen, 1909, 3, p. 334.

4. Ztschr. f. Immunitätsf., 1910, 7, p. 578.

Altho no difference occurred between the percentages of the positive cases in complement-fixation tests with leprous sera and either old tuberculin or leprous nodule, cases nevertheless occurred in which, not only were the results obtained in both tests reversed, but in certain syphilitic sera the leprous nodule antigen brought about a considerably large number of positive results. This fact appears to us to be explained on the ground that these results are non-specific in their nature, if the term "specific" be applied in its wider sense, inasmuch as the heart extract of a guinea-pig can be said to produce a "specific" reaction to syphilis. These facts led us to conjecture that this positive result may have been a reaction, not against the disease primarily present in the sera, but against certain other co-existing properties, a case in which the ratio of the genuinely positive results would have been less than that appearing in our table (see Table 9).

The clot of leprous blood, the sera of which reacted positively with leprous nodule antigen (leprous positive clot antigen), and the blood clot from a guinea-pig brought about identical results, as did the old tuberculin in complement-fixation tests with leprous sera; but we regret that they were tested in too few cases to enable us to draw any conclusions therefrom. The leprous negative clot antigen, which was obtained from the blood giving no complement-fixation with leprous nodule antigen, and the clot from a syphilitic patient, which gave negative results with the leprous nodule antigen, gave almost negative results, that is, identical with those produced in non-leprous (*viz.*, syphilitic) sera. It seems, therefore, that these antigens contain properties differing from those of the other two antigens. Closer observation, moreover, reveals the fact that there were some cases that gave a very slight positive reaction with leprous positive clot antigen, as well as with the negative clot antigen; for this reason we deem it necessary to continue the study of these two kinds of antigens.

FURTHER OBSERVATIONS ON THE SEROLOGIC DIAGNOSIS OF LEPROSY *

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In this report we intend to give the results of our subsequent experiments, made with a view to determining the consistency of our conclusions in regard to old tuberculin and extracts of guinea-pig heart. At the same time we shall quote an instance in which cases of syphilis were mistaken for leprosy.

The method of examination was substantially the same as that described in our previous report. The results of the examination are shown in Table 1.

TABLE 1
RESULTS OF THE COMPLEMENT-FIXATION TESTS WITH OLD TUBERCULIN AND WASSERMANN'S SYPHILIS ANTIGEN

Patient	Forms of Disease	Old Tuberculin		Wassermann's Test	
		1st.	2nd.	1st.	2nd.
1. M.	Mild anesthetic leprosy	+	±	+	+
2. F.	Light anesthetic leprosy	—	—	+++++	+++++
3. F.	Light anesthetic leprosy	+	±	+++++	+++++
4. M.	Light anesthetic leprosy	+++++	+++	+++++	+++++
5. F.	Light tubercular leprosy	±	—	+++++	+++++
6. M.	Mild anesthetic leprosy	—	—
7. F.	Mild anesthetic leprosy	+	—	+++++	+++++
8. M.	Mild tubercular leprosy	++	+	+++++	+++++
9. M.	Mild tubercular leprosy	+++	±	+++++	+++++
10. M.	Severe tubercular leprosy	+++++	+++++	+++++	+++++
11.....	Syphilis	—	—	+++++	+++++
12.....	Syphilis	—	—	+++++	+++++
13.....	Syphilis	—	—	+++++	+++++
14.....	Syphilis	—	—
15.....	Ulceration on the hip	—	—
16.....	Diagnosis not yet established	—	—
17.....	Syphilis	—	—	+++++	+++++

* Received for publication June 18, 1915.

If we summarize the results with old tuberculin alone, and compare them with what we have gathered from the first examination, we shall obtain the results in Table 2.

TABLE 2
THE STATISTICAL RESULTS OF THE TEST WITH OLD TUBERCULIN AS ANTIGEN

Diseases	Total Number of Examinations	Positive Cases	Percentage
Leprosy	39	27	69.23 first examination
Leprosy	10	7	70.00 second examination
Sum	49	34	69.38
Non-leprosy	8	0	0 first examination
Non-leprosy	7	0	0 second examination
Sum	15	0	0

Thus far it is seen that old tuberculin produced positive results in the complement-fixation test with the serum of leprosy, while with non-leprosy sera it gave negative results.

TABLE 3
THE STATISTICAL RESULTS OF THE TEST WITH WASSERMANN'S SYPHILIS ANTIGEN

Number of Examinations	Positive Cases	Percentage
24	17	70.84 first examination
9	9	100.00 second examination
33	26	78.79

From Table 3 it is seen that a strikingly large number of leprosy sera gave positive results with the Wassermann syphilis antigen, and that consequently it is necessary to take this fact into consideration in establishing a diagnosis of syphilis in a land where leprosy prevails to a tolerably great extent. We have verified the fact that, of 2,016 patients sent to the government leprosy hospital, seven were found to be syphilitics.

Leprosorium	Total Number of Cases Sent to Each Leprosorium	Syphilis Patients
First division Zen-sei-Leprosorium	723	3
Second division Hokubu-hoyoin-leprosy	225	2
Third division Soto-shima-leprosy	680	1
Fifth division Kiu-shiu-ryoyosho-leprosy	386	1
Total	2,014	7

While serologically leprosy has a certain degree of homogeneity with syphilis, as regards the Wassermann test at least, nevertheless it will not be wholly superfluous to try the complement-fixation test with old tuberculin in cases in which these two diseases are difficult to diagnose clinically.

CONCLUSIONS

In our first series, 69.23 percent of the total number of cases gave positive results with old tuberculin. In this second series, 70 percent were positive—practically the same result. If we add these two figures, we find that forty-nine cases were examined in all, of which thirty-four, or 69.38 percent, reacted positively.

On the other hand, eleven cases of syphilis, and four other cases of non-leprous diseases, that is, fifteen cases in all, gave negative results.

The fact that old tuberculin reacts specifically with leprous serum in the complement-fixation test, has thus again been demonstrated.

Thirty-three cases of leprosy were examined by the Wassermann test, of which twenty-six, or 78.79 percent, were positive. This shows that the Wassermann test brings about a greater percentage of positive results than does old tuberculin, for of the nine cases examined with both kinds of antigen, only seven produced positive results with the latter, while all of them were positive with the Wassermann test.

If, therefore, the serum of a doubtful case (whether syphilis or leprosy) produces positive results both with old tuberculin and the Wassermann antigen, the probabilities are that the patient is a leper. However, negative results with old tuberculin are not sufficient to exclude a diagnosis of leprosy. On this occasion we observed no case of leprosy that reacted positively only with old tuberculin.

Of the 2,016 cases sent to the Government leprosoria as lepers, seven cases were found by clinical observation to be syphilitic. Such cases cannot be subjected to the usual Wassermann method of differential diagnosis, but the complement-fixation test with old tuberculin as antigen will help greatly, for, altho this is inert for syphilis and many other diseases, it will produce positive results with the majority of leprous patients. The necessity of these methods arises from the difficulty which is sometimes met with in detecting the germ by microscopic examination.

IRITIS AND OTHER OCULAR LESIONS ON INTRAVENOUS INJECTION OF STREPTOCOCCI *

WITH PLATES 6-8

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In a previous paper¹ I pointed out that streptococci from rheumatic arthritis and myositis, when injected intravenously into animals, are prone to lodge in the capillaries of the iris, producing iritis and other lesions of the eye. Now, I wish to record further observations with illustrative experiments the results of which indicate that iritis and certain other lesions of the eye, generally thought to be toxic, may be infectious in nature.

As the outcome of a long series of experiments in which animals were injected intravenously under uniform conditions with streptococci from rheumatism, from appendicitis, from ulcer of the stomach, from cholecystitis, from erythema nodosum, from herpes zoster, from parotitis, from pyorrhea, from tonsils, and from dairy products, I have had opportunity to study the eye lesions in forty-eight animals. The lesions may be classified as follows: (1) unilateral panophthalmia in five instances (twice following injections of strains from rheumatism and herpes zoster as isolated, and once in each case after one animal passage, and once in a rabbit injected with a streptococcus mucosus from the eyeball in a case (for which I am indebted to Dr. Lane) of metastatic panophthalmia following tonsillitis and otitis media); (2) hemorrhages in the limbus, with or without episcleritis, in fourteen instances (four times following injection of laboratory strains after they had attained a certain grade of virulence, three times with rheumatic strains, twice with strains from appendicitis, twice with strains from herpes zoster, and once each with strains from erythema nodosum, myositis, and from butter); (3) iritis or iridocyclitis nine times (four times after injection of certain laboratory strains, three times with rheumatic strains, and twice following injection of streptococci from small pus pockets in tonsils of otherwise normal individuals); (4)

* Received for publication June 21, 1915.

1. Jour. Infect. Dis., 1914, 14, p. 61.

conjunctivitis, usually bilateral, occurring frequently, in ten instances associated with the streptococci injected (once each with streptococci from appendicitis, herpes zoster, and rheumatism, once with streptococci from butter, once with streptococci from the pus pocket in the tonsil of a cow, twice after injection of a laboratory strain, and three times after injection of strains from pus pockets in tonsils from approximately 100 individuals); (5) ulcer of the cornea due to the organism injected, observed only once, after injection of a strain of streptococcus from the muscle in calcifying myositis in a man. Lesions similar to these occurred with or without herpes in twelve animals injected with the strains from herpes zoster. Herpes of the conjunctiva followed injections of streptococcus from the tonsil in ulcer of the stomach in a cow. Hemorrhages in the retina were not often looked for, but were found in two instances.

It must not be supposed that the occurrence of these lesions is accidental because they appear to occur commonly only after injections of strains as isolated in rheumatic arthritis and myositis (17 percent) and in herpes zoster (15 percent), while after injections of strains from appendicitis, ulcer of the stomach, cholecystitis, and erythema nodosum, eye lesions are extremely rare. Moreover, strains from various sources may be made to acquire affinity for the eye. Thus, Strain R 51 A, which had been isolated eleven years previously as a pneumococcus and which had long since lost its virulence, was passed successively through eighteen animals without producing eye lesions in a single instance; but, in the eighteenth passage, it produced suppurative conjunctivitis in the only rabbit injected. In the nineteenth passage, it produced iritis (rabbit). In the twentieth passage, it produced suppurative conjunctivitis associated with iritis and marked hemorrhages and infiltration at the limbus in a dog, and marked conjunctival and scleral hemorrhages, especially at the limbus, in a rabbit. Subsequent passages failed to produce eye lesions. Exactly similar results were obtained from injection in successive animals of two strains of pneumococci, which at the time grew as streptococci, and which had lost their virulence by long cultivation on artificial media; each produced iritis in the tenth and fourteenth animal passage, while all the other injections failed to produce eye lesions. The injection of Strain 12, which originally had been isolated as a hemolytic streptococcus from "milk slime," but which had lost its hemolytic property and had acquired the properties of a typical streptococcus viridans by growth in symbiosis with *Bacillus subtilis* on blood agar plate, pro-

duced no eye lesions in the first seven injections, but in the eighth animal passage produced hemorrhages about the limbus and several in the retina in two rabbits injected, while in all subsequent passages no eye lesions developed. Finally, if the lesions in the eye really depend on a certain quality or virulence of the streptococci, then the associated lesions in the other organs should be similar, following injection of the different strains, at the time the eye lesions develop. This, on analysis, is found actually to be the case. Thus, of forty-eight animals showing eye lesions, twenty-eight showed arthritis, usually mild; five showed hemorrhages in the appendix; eight, ulcer of the stomach; ten, lesions of the endocardium; eight, of the pericardium; nineteen, of the muscles or fascia; and twelve, of the kidneys. The incidence and character of associated lesions in animals, therefore, correspond closely to what is observed in man. It must not be supposed, however, that the occurrence of eye lesions at a certain grade of virulence holds only for streptococci. Similar results have been obtained from injection of colon bacilli and staphylococci.

The lesions were usually acute and consisted of extravasation of red blood corpuscles and a variable infiltration of leukocytes. Suppurative iritis was observed only once (see Fig. 1). In the instances of panophthalmia, there was turbidity of the aqueous humor, which contained leukocytes and from which the organisms injected could usually be isolated in pure culture.

The organism injected was demonstrated in the lesions in large or small numbers by cultural methods and in sections in a large number of instances, sometimes after the animals had recovered and the blood was sterile. In fact, so constantly was the organism injected found that one feels almost justified in concluding that the occurrence of a localized hemorrhage, for instance, means the presence in it of bacteria. This holds true, not only in the case of streptococci, but in that of staphylococci and colon bacilli as well.

The bacteria were found within blood vessels adjoining the lesions and in the areas of hemorrhage and infiltration, usually with aggregation of leukocytes and endothelial cells. At times, when the animal survived the injection, active phagocytosis by leukocytes and endothelial cells could be made out. Smears from the pus in the conjunctival sac in instances of conjunctivitis which were observed in rabbits, dogs, and one monkey, and in the instances of panophthalmia, commonly showed diplococci within and without leukocytes. Plate cultures on blood agar and fermentative tests were used to estab-

lish the identity of the organisms injected and of those found in the inflamed conjunctival sac. The cultures, made after repeated washing of the muco-purulent material in salt solution, at times yielded the organism injected in pure culture; usually, however, together with a variable number of other bacteria, mostly saprophytic bacilli. Not all instances of conjunctivitis were due to the streptococci injected. The frequent occurrence of lesions about the ciliary body or the iris and the limbus needs special emphasis. This might be thought to be due to the fact that here, as in the structure about the joints and the more tendinous portion of muscles, there is a gradation from an abundant to a very scanty blood supply, and hence a gradation of the supply of available oxygen, thus inviting localization and affording opportunity for the growth of bacteria, a circumstance which for the same reasons would make for lesions by circulating toxic substances, no matter from what source.

The following experiments will serve to illustrate the results obtained:

RABBIT 581.—Large, black rabbit, injected intravenously, Dec. 31, 1913, with the growth from 45 c.c. of ascites dextrose broth of *Streptococcus* 839, which had been isolated from under the crust of a crushing injury of the thumb in a case of acute rheumatic fever with pericarditis in a young man 19 years old.

Jan. 3, 1914.—The rabbit died at 12 noon. Examined at once: Marked circumcorneal congestion of the left eye; fluid in the anterior chamber distinctly turbid; anterior portion of the iris covered with a plastic, slightly adherent exudate, smears of which showed leukocytes and numerous diplococci; the lens and other structures of the eye apparently normal; the right eye normal; the joint and pericardiac fluids distinctly turbid and increased in amount; a number of whitish areas associated with hyperemia and hemorrhage in the muscles, especially in the subscapular muscles of the right side and in the muscles of the spine; one rather large hemorrhage in the mucous membrane of the pylorus of the stomach; spleen distinctly enlarged, and hyperemic; no other gross lesions.

Jan. 4, 1914.—Cultures from the blood showed a few streptococci; cultures from the pericardiac and joint fluids gave a moderate number, while cultures from the fluid in the anterior chamber of the left eye gave an enormous number of colonies of streptococci only, which produced a slight hazy zone of hemolysis on blood agar plates; cultures of the fluid in the anterior chamber of the right eye, sterile. Sections of the eye showed marked necrosis and leukocytic infiltration of the iris, most marked over the anterior layer where there were very many diplococci (see Figs. 1 and 2).

RABBIT 61.—Medium-sized Belgian hare, injected intravenously, March 4, 1914, with the growth from 45 c.c. of ascites dextrose broth of a streptococcus isolated from a small quantity of pus expressed from the tonsil ten days after an attack of recurring herpes involving the thigh.

March 6.—Seemed ill. Chloroformed and examined at once: Two sharply circumscribed areas of hemorrhage and edema of the right eye, one (0.3 cm.) directly opposite the attachment of the iris near the limbus, the other (0.5 cm.)

situated just where the external rectus merges into the sclera (Fig. 3); the blood vessels surrounding these areas showed congestion; the iris showed no visible changes; the fluid in the anterior chamber clear; moderate increase in amount and turbidity of the joint fluid in both knees and wrist joints; intussusception of ileum associated with plastic adhesions; no other focal lesions.

March 8.—Cultures made from the blood, joint fluids, and fluid in the anterior chamber of the right eye were sterile. Sections of the eye showed subscleral hemorrhages associated with beginning round cell infiltration just outside of the attachment of the iris in the ciliary body. A thorough search for bacteria in gram-stained sections showed a few diplococci, free and within leukocytes and endothelial cells, one of which is shown in Fig. 4.

RABBIT 70.—Medium-sized, white rabbit, injected intravenously, March 4, 1915, with the growth from 15 c.c. of ascites dextrose tissue broth of a streptococcus mucosus in the second culture, isolated from the eyeball during recovery from metastatic panophthalmia in a middle-aged man.

March 6.—Seemed well; marked lacrimation and circumcorneal injection of blood vessels of the left eye; the cornea opaque, and the fluid in the anterior chamber turbid. Chloroformed: A whitish-gray, radiating deposit on the anterior surface of the iris, smears from which showed many leukocytes and other cells and a moderate number of diplococci with a distinct capsule; no other lesions; sections of the iris and adjacent structures showed hyperemia and moderate leukocytic infiltration, but unfortunately the film of exudate was largely lost in the preparation. However, a search for bacteria showed a number of diplococci near the attachment of the iris and a short chain of diplococci in the anterior chamber just outside an area of hemorrhage in the iris (Fig. 5).

RABBIT 945.—Small, white rabbit, injected intravenously, Nov. 23, 1914, with the growth from 15 c.c. of dextrose broth of a colon bacillus isolated from the wall of the appendix in a case of subacute appendicitis.

Nov. 24.—Found dead: Numerous hemorrhages in the conjunctiva and in the limbus of right eye (Fig. 6); marked subperitoneal and mucous hemorrhages throughout the appendix; moderate hemorrhages in Peyer's patches and mucous membrane of the duodenum, colon, and sigmoid and in common and hepatic ducts and apex of the gall-bladder; a few punctate hemorrhages in the myocardium and skeletal muscles.

Nov. 25.—Cultures from blood and areas of hemorrhage showed large numbers of colon bacilli. Sections of the hemorrhagic area in the eye showed a large number of bacilli with varying degrees of decolorization when stained by Gram's method (Fig. 7).

EXPLANATION OF PLATES 6-8

Fig. 1.—Section of iris and ciliary body of rabbit showing marked leukocytic infiltration four days after an intravenous injection of streptococcus from rheumatic arthritis. Hematoxylin and eosin. $\times 60$.

Fig. 2.—Streptococci in area of infiltration in Figure 1. Gram-Weigert stain. $\times 1200$.

Fig. 3.—Localized hemorrhages in the sclera near the limbus and at the attachment of the external rectus muscle in rabbit forty-eight hours after an intravenous injection of streptococcus from pus pocket in tonsil. $\times 3\frac{1}{2}$.

Fig. 4.—Diplococcus adjacent to area of hemorrhage in Figure 3. $\times 1200$.

Fig. 5.—Diplococci in anterior surface of iris just opposite an area of hemorrhage into the iris. See Rabbit 70. Gram-Weigert stain. $\times 1200$.

Fig. 6.—Section of limbus of eye in rabbit showing hemorrhage and slight leukocytic infiltration twenty-four hours after intravenous injection of colon bacilli. Hematoxylin and eosin. $\times 430$.

Fig. 7.—Colon bacilli in varying stages of decolorization in area of hemorrhage shown in Figure 6. $\times 1200$.

PLATE 6

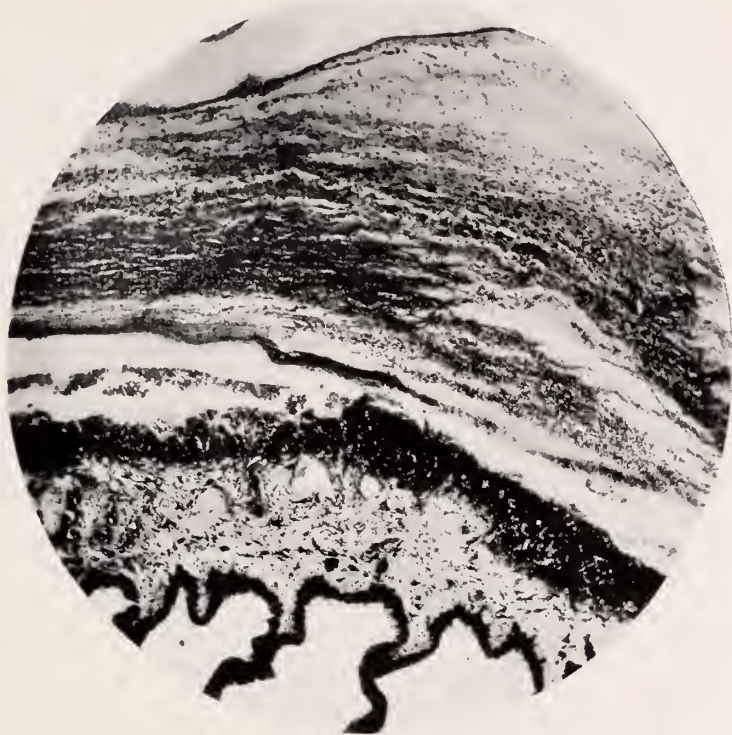


Figure 1.

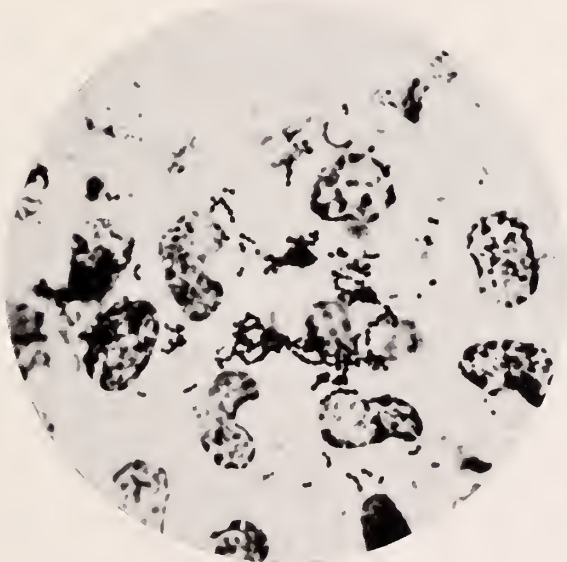


Figure 2.

PLATE 7



Figure 3.



Figure 4.



Figure 5.

PLATE 8

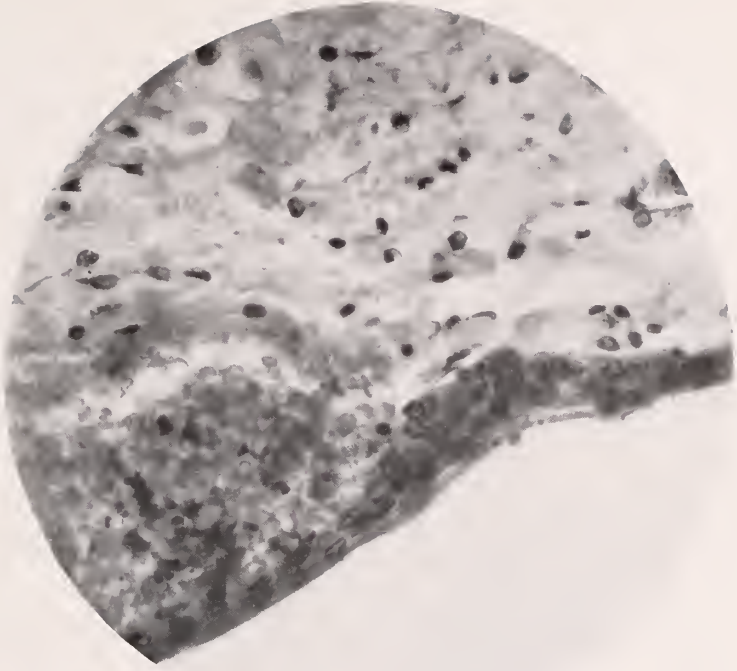


Figure 6.

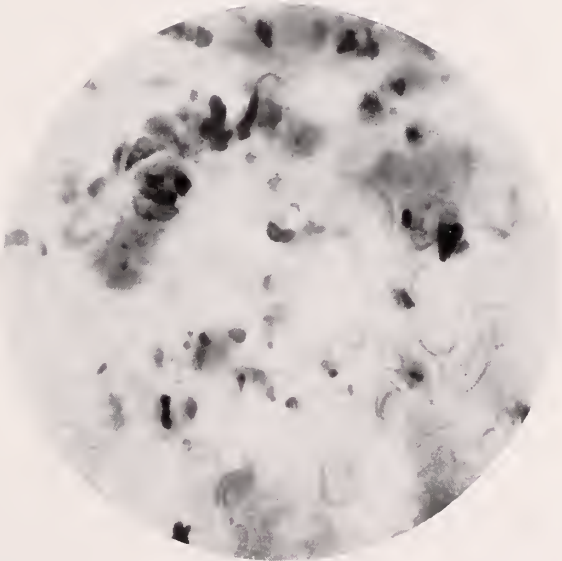


Figure 7.

THE EFFECT ON ANTIBODY PRODUCTION OF THE REMOVAL OF VARIOUS ORGANS *

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Various methods have been used in the study of the problem of where in the body the antibodies are formed that develop in response to immunization. The principal methods are: (1) The determination of the tissues in which the newly formed antibodies are demonstrable before they appear in the blood; (2) the determination of the tissues in which the antigenic substances become fixed; (3) the study of the effects on antibody production of the removal of organs, limited so far almost entirely to splenectomy; (4) the study of the effects of various substances, e. g., arsenical compounds, benzene, etc., of acute anemia, and of the x-ray on antibody fabrication; and (5) the study of the production of antibodies by tissues under cultivation *in vitro*.¹ It is not necessary to say more about the results at this time than that they all point directly to the spleen, the marrow, and the lymphatic tissues as the centers for the manufacture of antibodies. The question arises what influence, if any, on antibody formation is produced by procedures such as the removal of various organs. Through observations of this nature, we possibly may obtain a little better understanding of the very remarkable process of the formation of antibodies.

We have had the opportunity to study the course of specific antibodies educed by the injection of rat blood into dogs from which important organs had been removed by surgical operations. While the material at our disposal was not so large and varied as might be wished, yet the results obtained are not without interest.

It is necessary, in order to determine accurately the effect that certain procedures may have on the production of antibodies, to use reliable standards of comparison. This is particularly true in the case of experiments that might lead to increase or diminution of the total amount of antibodies produced in response to the introduction of fixed quantities of antigen. As the curves described by the increase and

* Received for publication June 22, 1915.

1. The literature is reviewed by Gay and Rusk, *Tr. Fifteenth Internat. Cong. Hyg. a. Demogr.*, 1912, 2, p. 328.

decrease of newly formed antibodies in the blood serum of different animals seldom run exactly parallel even under the most strictly comparable conditions, isolated measurements, even if made at the time the concentration usually reaches its highest point, do not constitute a safe basis for comparisons from which to draw conclusions. The only safe way is to make frequent determinations so that complete curves can be drawn and compared with the normal standards in each case, namely, the complete course of antibodies in a considerable number of normal animals injected in the same way with the same quantities of antigen. This is the plan we have followed.

From a large series of observations, it has been determined that in adult dogs in ordinary health the injection intravenously of 1 c.c. of a 10 percent suspension of rat blood per kilo of weight is followed by a wave-like production of antibodies that runs a fairly typical and constant course. The particular bodies are the specific opsonin and agglutinin, which run parallel, lysin and precipitin apparently not being produced, except in very small amounts, while no tests have been made for bodies that fix complement. For a day or two after the injection, there sometimes is a fall in the agglutinin and opsonin for rat corpuscles normally present in the serum of the dog. The rise usually begins on the fourth day after the injection, sometimes a day or so later, but very rarely, if ever, earlier. The highest point is reached on the twelfth day as a rule, occasionally a little earlier, hardly ever later. In a few days, a gradual diminution begins, the normal level being reached or closely approached at about the fortieth day after the injection. This general course of the antibodies is illustrated in Table 1. The high point does not seem ever to go beyond a dilution of the serum of 7 to 6144, and very rarely indeed has it been found below a dilution of 1 to 1536.

The measurements are made by finding the highest dilution in which the serum causes undoubted agglutination or opsonification of rat corpuscles in a mixture of 0.6 c.c., containing 0.2 c.c. of a 5 percent suspension of corpuscles, carefully washed, the rest being dog serum and salt solution in the case of the agglutinin tests, and dog serum, salt solution, and a suspension of washed leukocytes in the case of the opsonin tests. The leukocytes are obtained from the pleural exudate of a dog, caused by injecting aleuronat. The mixtures are incubated one hour, and then the results are determined, in the case of the

opsonin tests, by the observation of phagocytosis in properly prepared, stained smears.

In experiments of this kind, special care must be used to exclude dogs with pneumonia (distemper?), because in several instances it has been found that the development of pneumonia in otherwise healthy

TABLE 1
THE EFFECT OF THE REMOVAL OF THE SMALL INTESTINE AND OTHER ORGANS IN DOGS ON THE DEVELOPMENT OF ANTIBODIES FOR RAT CORPUSCLES

Days After Injection of Rat Blood	Control Animal. No Surgical Interference	Removal of Small Intestine 2 Days Before Injection of Rat Blood	Removal of Pancreas and Spleen 2 Days Before Injection of Rat Blood	Removal of Spleen 4 Days After Injection of Rat Blood	Removal of Pancreas 4 Days Before Injection of Rat Blood	Removal of Pancreas 5 Days Before Injection of Rat Blood	Removal of Stomach 7 Days Before Injection of Rat Blood
1	48	48	48	48	96	96	48
2	48	48	48	48	96	96	48
3	48	96	48	48	96	96	48
4	48	96	96	192	192	384	96
5	192	96	96	384	192	384	192
6	768	96	192	768	384	384	192
7	1536	96	384	768	384	384	384
8	1536	96	768	1536	384	384	384
9	1536	192	768	384	384	384	1536
10	3072	384	..	384	Death from intussusception	384	1536
11	3072	768	768	384		384	3072
12	3072	768	768	192		384	3072
13	3072	768	1536	192		384	3072
14	3072	768	768	192		384	1536
15	3072	1536	768	192		384	1536
16	3072	1536		192		384	1536
17	1536	1536				384	768
18	1536	1536				384	768
19	1536	1536				384	768
20	1536	1536				384	768
21	1536	768				384	384
22	1536	768				384	
23	1536	768				384*	
24	1536	768				192	
25	1536	768				96	
26	768	384				96†	
27	768	384					
28	768						
29	768						
30	768						
31	768						
33	768						
36	384						
39	192						

The figures give the highest dilution of the serum causing definite agglutination and opsonification of rat corpuscles.

* Rabies. † Death.

animals at or about the time of injection of the antigen has inhibited completely or almost completely the formation of antibodies. In this series of experiments, we are confident that pneumonia did not play any part.

The effect of the removal of the stomach, of the pancreas, of the pancreas and the spleen, of the small intestine below the duodenum,

and of other operative procedures on the production of antibodies to rat corpuscles has been studied. In practically all the animals injected soon after the operation, there developed a fairly typical antibody curve, in some cases of rather low range, and in others with some prolongation of the first phase.

Complete removal of the stomach resulted in practically no change in the usual curve after the injection of rat blood.

In one dog from which the pancreas had been removed, death occurred on the thirteenth day, probably on account of an intussusception which had existed for some days. In this animal the amount of antibody produced was much smaller than usual (Table 1). In the case of another dog that was injected with 1 c.c. 10 percent rat blood per kilo on the fifth day after removal of the pancreas, the resulting production of antibody was also comparatively small, but in this case rabies developed and caused death on the twenty-seventh day after the operation (Table 1). It is consequently possible that in this instance the rabic infection may have interfered with the antibodies, altho in experiments on dogs infected with rabies but not subjected to the removal of any organs there seemed to be no diminution in antibody production.

The simultaneous removal of the spleen and pancreas did not reduce the elevation of the curve any more than removal of the spleen alone at about the time of injection of the antigen (Table 1).

Da Costa and Beardsley² found that in diabetic patients the opsonins for streptococci, staphylococci, and tubercle bacilli are reduced very much, as measured by the opsonic index, and Thomas and McPhail³ record that, in pancreatectomized dogs, injection of *Staphylococcus aureus* was not followed by increase in the opsonic index, whereas, in normal dogs, such injection was followed by increase in the index. From these results, which accord well with the diminished resistance of diabetics to infection, the investigators quoted conclude that resistance to infection depends, at least in part, on the activity of the pancreas. As they made their observations in the usual way, that is, with unheated serum, it is possible that the lower index in diabetics and in pancreatectomized dogs results from diminution in the thermolabile opsonic element—complement—rather than from failure of the thermostable element to increase. On this account their results may not warrant the conclusion that in diabetes the power to produce antibodies

2. *Am. Jour. Med. Sc.*, 1908, 136, p. 361.

3. *Proc. Path. Soc.*, Philadelphia, 1911, 14, p. 108.

in diminished greatly. Handman⁴ argues that it is the local resistance which is diminished in diabetes, and not the power to form antibodies, but does not produce any experimental evidence in favor of this view. It is unfortunate that, in our experiments, the results of which point to a loss in the power to produce antibodies, we are unable to exclude the possible action of complicating conditions, so that further experiments are required to settle the question definitely.

Removal of the small intestine was made in two dogs. One was injected with rat blood two days later. In both there resulted an average amount of antibody as determined in the usual way; but in the first dog the period of latency was nine days, while in the second it was only four days (Table 1).

Ligation of the mesenteric artery previous to injection of the antigen was followed by a somewhat longer period of latency than usual and a rather small amount of antibody.

When the amount of lymphatic tissue removed in connection with removal of the small intestine or affected by circulatory disturbances of the small intestine, is considered, it hardly seems difficult to understand that the same general effect might follow as seen after removal of the spleen; namely, more or less reduction in the amount of antibody produced. Any cause of the prolongation of the latent period is however not evident.

We have had no opportunity to follow the effects on the antibody curve after removal of parts of the liver in dogs, but in white rats removal of one-half of the liver about twenty-four hours after the introduction of the antigen (sheep blood) was without any demonstrable effect on the amount of lysin produced.

In dogs with Eck's fistula (established by Dr. Miller), the intravenous injection of the usual amount of rat blood was followed by a typical antibody curve. The establishment of the fistula in no way affected the normal antibodies. The observations were all made soon after the fistula was established, and while the animals all appeared to be in good health.

Adrenalectomy in normal dogs, and in dogs at the height of the antibody curve after the injection of rat blood, did not cause any fall in the antibody content of the blood serum, as determined by hourly observations after the operation and until death.

4. Deutsch. Arch. f. klin. Med., 1911, 102, p. 1.

Thyroidectomy immediately after the injection of the antigen, death from tetany occurring on the tenth day, was not followed by any variation in the usual course and amount of antibody production up to that day. This result is in harmony with the results obtained by Fjeldstad⁵ in rabbits, in which thyroidectomy had no influence on the production of agglutinins for typhoid bacilli. Our result is also in harmony with the observation of Launoy and Levy-Bruhl⁶ that in chickens thyroidectomy has no influence on the development and course of spirochetosis gallinarum.

SUMMARY

In the dog, complete removal of the stomach, of the small intestine, or of the thyroid does not interfere with the development of the agglutinin and opsonin for rat corpuscles after intravenous injection of rat blood.

Pancreatectomy, complicated by intussusception and by rabies, resulted in a diminution in the amount of antibodies that accumulated in the blood.

Simultaneous removal of spleen and of pancreas appears to have about the same depressing effect on antibody production in dogs as removal of the spleen only.

Removal of the small intestine and ligation of the mesenteric artery before the injection of rat blood in dogs may be followed by a longer latent period after the injection of antigen than in normal dogs.

Adrenalectomy at the height of the antibody curve in dogs does not change the antibody content of the blood serum.

In rats, removal of about one-half of the liver appears to have no effect on the development of lysin for sheep corpuscles.

The results recorded indicate that the mechanisms for the fabrication of antibodies are quite secure from certain disturbances, and they are in no way contradictory of the current view that these mechanisms are located in the blood-forming organs.

5. *Am. Jour. Physiol.*, 1910, 26, p. 72.

6. *Ann. de l'Inst. Pasteur.*, 1915, 29, p. 213.

THE INFLUENCE OF THE X-RAY ON THE PRODUCTION OF ANTIBODIES *

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Heinecke and others¹ have shown that in proper doses the x-ray has a direct and destructive action on the lymphoid and myeloid tissues. The destruction begins very soon after the exposure; the lymphatic glands and follicles appear to be affected first, next the spleen, and then the bone-marrow. The lymphocytes in the thymus are quickly destroyed by the x-ray.² The cells undergo direct necrosis with nuclear fragmentation, and, if the process is made to take place gradually, destruction of the lymphoid cells and the myelocytes may be accomplished without appreciable effect on other tissues or on the general health of the animals. The leukocytes in the circulating blood are reduced in number, and, when this effect is marked, the mononuclears proportionately more than the polymorphonuclears.³ When the action of the ray is at an end, regeneration of cells and reconstruction take place.

In view of this action on the hematopoietic system, the question arises what effect has the x-ray on the production of antibodies? This question has received some attention and attempts have been made to study it along two principal lines, namely, by the direct study of the development of antibodies in animals treated with the x-ray and by observations on the resistance of x-rayed animals to infection. Benjamin and Sluka⁴ found that in rabbits exposure to the x-ray before the injection of beef serum diminished very much the production of specific precipitin or prevented its formation altogether at the same time as there resulted, in some cases, a considerable delay in the disappearance of the antigen from the blood of the injected animals. If applied four days or so after the injection of beef serum, or at the

* Received for publication June 6, 1915.

1. Heinecke, Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1905, 14, p. 21; Warthin, Internat. Clin., 1905, 4, p. 243; Murphy and Ellis, Jour. Exper. Med., 1914, 20, p. 397.

2. Rudberg, Thymusinvolution efter Röntgenbestrålning, Upsala, 1909.

3. Concerning changes in blood in workers with the x-ray, see Portis, Jour. Am. Med. Assn., 1915, 65, p. 20.

4. Wien. klin. Wchnschr., 1908, 21, p. 311.

height of precipitin production, however, the ray was without any effect on the precipitin. Låwen⁵ observed that the formation of specific bacterial agglutinin and lysin was restrained in animals treated with the x-ray, and that no action was had on agglutinin in the blood of normal animals or on typhoid agglutinins subjected to exposure in vitro.

Von Heinrich⁶ studied the effects of the x-ray on the phenomena of anaphylaxis in guinea-pigs. He found that animals given a single exposure of x-ray ("3 Kalom, 1 Erythemdosis") soon after the primary injection of antigen, reacted very much less violently on re-injection of the antigen about fourteen days later than did animals which were not exposed to the x-ray. Furthermore, passive anaphylaxis could not be produced with the serum of guinea-pigs treated with the x-ray soon after injection of antigen; hence, it would seem logical to describe the action of the x-ray as interference with the production of the specific body on which anaphylactic shock depends. This view is supported by the fact that the x-ray appears to have no effect on the complement. In animals that received the second injection some six weeks after the primary injection, which had been followed by exposure to the x-ray, the reactive phenomena are described as especially severe. Von Heinrich suggests that the antigen may have remained in available form so long that when regeneration of the blood-making tissues set in after the x-ray injury, specific antibody was produced. This explanation is in accord with the observation of Benjamin and Sluka that the antigen persists longer in the blood of rabbits treated with the x-ray than in the blood of rabbits not so treated.

Buckner⁷ tried to stimulate, by means of exposures to x-ray, the production of specific antibodies in mice injected with trypanosome vaccine, but the results obtained were not constant.

Of the experiments designed to test the effect of the x-ray on the resistance of animals to infection, the earliest ones were based on the idea that, on destruction of leukocytes by the x-ray, substances would be set free that would protect the animal.⁸ Under the conditions of certain experiments, indications of increased resistance were obtained, but as yet we have no confirmatory reports. On the other hand, Låwen

5. Mitt. a. d. Grenzgeb. d. Med. u. Chir., 1909, 19, p. 141.

6. Centralbl. f. Bakteriöl., I, O., 1913, 70, p. 421.

7. Ztschr. f. Hyg. u. Infektionskrankh., 1913, 73, p. 411.

8. Quadroni, Centralbl. f. in. Med., 1905, 26, p. 521; Heile, Ztschr. f. klin. Med., 1904, 55, p. 508; Helber and Linser, München. Med. Wchnschr., 1905, 52, p. 689.

was unable to detect any changes in the blood serum or any setting free of endoleukocytic enzymes after destruction of leukocytes by means of the x-ray, and he did find that the resistance to various bacteria on the part of rats, mice, and guinea-pigs was reduced greatly thereby. More recently, Murphy and Ellis⁹ find that after exposure to the x-ray mice (normal and splenectomized) became more susceptible to bovine tuberculosis than normal animals. X-ray, in the doses they used, affected only lymphoid tissue, and this result in conjunction with other facts leads them to regard the lymphocyte as an important agent of defense against tuberculosis.

In connection with other experimental work on the production of antibodies under various conditions, it has been found desirable to study the effect of the x-ray also. For this purpose, the white rat has been used, and the antibody observed is the specific lysin that develops after the injection of sheep blood. The exposures to the x-ray have been made in the x-ray laboratory of the Presbyterian Hospital of Chicago and I am under much obligation to Dr. Hollis E. Potter and Dr. J. W. Rowntree.

EXPERIMENT 1.—Twenty-five rats, of about the same age, weighing from 60 to 80 grams were used.

The distance from the tube to the rats was 18 inches. Secondary current, 4 milliamperes. Tube backing up 5 inches parallel spark-gap. Time of each exposure, eight minutes.

Immediately after the first exposure, the rats were injected intraperitoneally with 5 c.c. per kilo of weight of a 10 percent suspension of sheep blood. Subsequently, the rats were given the same exposure to x-ray daily. After the second day, two rats were killed every day or every other day, the leukocytes counted, and the titer of the lysin for sheep corpuscles in the serum determined. After the fifteenth day, only one rat was killed at a time. In no case did the general health seem to be affected. The thymus especially and the spleen were usually diminished in size and often the lymph nodes were reduced to mere traces.

The determination of the lysin was made by finding the highest dilution of the serum, heated at 56 C. for thirty minutes, and complemented with guinea-pig serum (0.0125 c.c.), at which distinct lysis was produced. The total quantity in each tube was 0.6 c.c., including 0.2 c.c. of a 5 percent suspension of sheep corpuscles, carefully washed, 0.0125 c.c. of guinea-pig serum, and the requisite amount of heated rat serum, the rest being salt solution. The mixtures were incubated for two hours and then placed in the ice-box until the following morning.

The results of the measurements of the lysin and the leukocyte counts are given in Table 1. The figures under "Controls" give the titer of the lysin of twenty-five rats of the same age and weight, treated in exactly the same way as the x-ray rats, except that they were not exposed to x-rays. In all cases, the figures under "Lysin" represent the highest dilution of the serum in which definite lysis occurred.

TABLE 1

THE LYSIN AND LEUKOCYTES IN EXPERIMENT 1 AND THE LYSIN IN THE CONTROL RATS

Days After First Expos- ure and Injection of Antigen	X-ray Rats				Lysin in Controls	
	Lysin		Leukocytes			
2	96	96	2,200	1,800	192	192
4	1,536	384	2,800	2,550	6,144	12,288
5	6,144	6,144	2,450	2,600	12,288	12,288
6	3,072	6,144	2,400	2,050	12,288	6,144
7	12,288	3,072	2,100	2,000	6,144	3,072
8	1,536	192	2,100	2,050	12,288	6,144
9	1,536	12,288	1,850	1,900	768	384
10	1,536	3,072	1,800	1,800	1,536	6,144
12	3,072	1,536	1,800	1,650	3,072	3,072
15	768	768	1,700	1,500	1,536	1,536
16	192	1,600	768
17	384	1,552	768
18	192	1,600	192
20	48	192
22	48	192

TABLE 2

THE LYSIN AND LEUKOCYTES IN EXPERIMENT 2

Days After First Exposure	Days After Injection of Antigen	Lysin		Leukocytes	
3	2	384	384	3,600	3,150
4	3	384	384	2,350	2,700
5	4	384	384	2,000	1,750
6	5	12,288	1,536	2,100
7	6	12,288	12,288	1,800	2,050
8	7	12,288	12,288	2,700	2,100
10	9	12,288	3,072	1,650	1,800
11	10	1,536	6,144	1,500	1,450
12	11	1,536	1,450
14	13	1,536	384	1,050	1,400
17	16	192	384	1,300	1,150
19	18	384	950

The results show a prompt reduction in the number of leukocytes in the rats exposed to the x-ray, differential counts showing the polymorphonuclear leukocytes to be relatively increased. The results do not indicate, however, that the exposures restrained in any noteworthy degree the formation of lysin in the x-ray rats as compared with the controls. Possibly the formation of lysin was checked somewhat in the beginning in the x-ray animals, and the figures point also to a more rapid fall in them, but the differences are hardly marked enough not to be explainable by individual variations and errors of observation. As the figures for each day represent the rats killed that day, individual variation must be considered in judging of the results.

EXPERIMENT 2.—In this experiment, twenty-two rats, of about the same age, weighing from 60 to 80 grams each, were exposed to x-ray under the same general conditions as in Experiment 1, but the length of each exposure was twice as long as in the first experiment, that is, sixteen minutes. The injection of sheep blood was made after the second exposure. The results are given in Table 2.

In this experiment, also, the results do not indicate that the effects of the x-ray in any considerable degree restrained the production of lysin, even tho the number of leukocytes was greatly reduced. On comparing the amounts of lysin in the serum of these rats with the amounts in the serum of the control rats in Experiment 1, it appears that at least during the first five days the lysin production remained considerably below that in the control animals.

In the next experiment, the rats were brought thoroughly under the influence of the x-ray before injecting the antigen.

EXPERIMENT 3.—In this experiment, nineteen young rats were x-rayed as in Experiment 1 for ten days before sheep corpuscles were injected. In the meantime, the leukocytes ranged around 3,000-4,000. The exposures were then continued, one rat being killed each day. In this series, also, the exposures were without any noticeable effect on the general condition of the rats. The results are given in Table 3.

TABLE 3
THE LYSIN AND LEUKOCYTES IN EXPERIMENT 3

Days After First Exposure to X-ray	Days After Injection of Sheep Blood	Lysin	Leukocytes
11	1	96	2,100
12	2	192	
13	3	96	2,250
14	4	96	2,100
16	6	96	2,200*
17	7	3,072	2,550
18	8	3,072	1,800
19	9	768	2,400
20	10	96	2,550**
21	11	1,536	1,750
23	13	48	1,800
24	14	96	1,450
25	15	48	1,950***

* Granulocytes, 80%; lymphocytes, 20%.

** Granulocytes, 53%; lymphocytes, 47%.

*** Granulocytes, 78%; lymphocytes, 22%.

This result was practically duplicated in Experiment 4, in which the rats were x-rayed for some days before the introduction of antigen and then continued, show that the formation of lysin was restrained very markedly. It is noteworthy that the total number of leukocytes is not depressed

any more than in Experiments 1 and 2; the relative decrease of mononuclears is marked, however.

This result was practically duplicated in Experiment 4, in which the exposure to the x-ray was not continued so long as in Experiment 3.

EXPERIMENT 4.—In this experiment, sixteen young rats were x-rayed as in Experiment 1 for five days and then given a double exposure for five days, sheep blood being injected intraperitoneally on the eighth day from the beginning. Two rats were killed each day from the second day after injection of sheep blood to the thirteenth inclusive; the three remaining animals were killed as shown in Table 4.

TABLE 4
THE LYSIN AND LEUKOCYTES IN EXPERIMENT 4

Days After First Exposure to X-ray	Days After Injection of Sheep Blood	Lysin	Leukocytes		
			Total	Granulocytes	Lymphocytes
8	2	24	1,400	44	56
		0	1,500	66	34
9*	3	0	900	40	60
		24	600	50	50
10	4	0	2,000	50	50
		48	700	40	60
11	5	96	700	40	60
		96	1,100	50	50
12	6	0	1,000	55	45
		0	1,000	55	45
13	7	24	1,100	56	44
		384	1,450	52	48
14	8	768	1,400	40	60
15	9	768	1,300	40	60
17	11	768	1,900	34	66

* Last exposure to x-ray.

In the rats of Experiment 4 examined before the seventh day after the injection of sheep blood, the spleen was very small, the thymus greatly reduced, and the lymph glands apparently absent, the bone-marrow being softer and more deeply red than normally. In the last three rats, the spleen was not so small and the thymus showed distinct islands of regeneration. The presence of newly formed lysin in the rats killed on the fourteenth, fifteenth, and seventeenth days may be accounted for by assuming that in these rats lysin production was not restrained very much and that when killed the course of the lysin had passed the high point on the return to normal conditions; or we

may assume that the lysin was the result of more recent activity by the centers of antibody production, antigen (sheep blood) remaining in available form until the centers had recovered from the effects of the x-ray. The second suggestion coincides with v. Heinrich's explanation of the severe anaphylactic reaction in guinea-pigs six weeks or so after the primary injection, followed by treatment with x-ray.

EXPERIMENT 5.—This experiment illustrates the result from a single exposure of twenty minutes, 10 milliamperes through Coolidge tube, focal distance 7 inches, spark-gap 5 to 6 inches, the antigen being injected immediately afterwards, each animal being bled from the heart on the fourth day or so and thereafter at intervals of two to three days while alive. Most of the rats so treated would die in eight to ten days with the usual symptoms of profound x-ray effects and as a rule with no, or very little, new production of lysin, the number of leukocytes being reduced very much, in some cases 400-500. In the animals that survived longer, some little lysin might appear in the blood after a longer period of latency than was observed in rats not exposed to x-ray.

TABLE 5

THE LYSIN AND LEUKOCYTES IN RATS EXPOSED TO X-RAY FOR TWENTY MINUTES BEFORE INJECTION OF ANTIGEN

Days After In- jection of Antigen	Rat 1		Rat 2		Rat 3		Rat 4	
	Lysin	Leuko- cytes	Lysin	Leuko- cytes	Lysin	Leuko- cytes	Lysin	Leuko- cytes
4.....	48	—	48	1,500	0 (24)	1,150	0 (24)	—
6.....	48	3,000	192	500	48	400	96	800
8.....	48	—	1,536	1,100	96	600	192	—
10.....	Died	Died	Died
11.....	1,536
12.....	Died

In this experiment, some of the animals showed no indication of lysin formation for eight days after the injection of the antigen; in others, there resulted production of antigen after a much longer period of latency than was the case in normal rats (see Table 1).

In the serum of the rats that failed to produce lysin after exposure to the x-ray, at least as judged by the tests, it is possible that inhibitory or antilytic substances were present. That such was the case does not seem likely, however, because the serum of x-rayed animals had no inhibitory effect whatever on the lysin in the serum of animals not treated with the x-ray or treated as in Experiments 1 and 2. The two kinds of serum were mixed in varying proportions and incubated for twenty-four hours, but no antilytic effect was obtained even in mixtures of one part of lytic serum to 192 parts of non-lytic x-ray serum.

Experiments were made to test the effect of the x-ray on the course of lysin in rats after its production was well under way. The results obtained are not conclusive. In order to study this point satisfactorily, it is necessary to bleed the same animal several times and this is not possible in the case of rats which have been exposed freely to the x-ray, as they do not stand repeated punctures of the heart. For this reason, further consideration of the effect of the x-ray on the course of antibodies is postponed until it can be studied in a larger animal and with reference also to other antibodies.

SUMMARY

Repeated exposures of white rats to the x-ray immediately after the injection of sheep blood do not interfere in any noteworthy degree with the production of lysin when the exposures, tho sufficient to reduce the number of leukocytes in the blood, have no definitely detrimental effect on the general health of the animals.

When the exposures are commenced some days before the injection of sheep blood and continued in such manner that the number of leukocytes is greatly reduced for fifteen to twenty days after the injection of sheep blood, the formation of lysin is restrained to a marked degree. This may be accomplished without any appreciable effect on the general health. In such animals the spleen, lymphatic tissues, and thymus are greatly reduced in volume and the bone-marrow changed. In the blood, the granular leukocytes are increased relatively and the total number of leukocytes is much below normal.

A single prolonged and eventually fatal exposure to the x-ray immediately before the injection of sheep blood may prevent any formation of lysin for the next eight days or so, or lysin may be produced after a longer period of latency than in the control animals.

The results harmonize with the view that antibodies are produced in the spleen, lymphatic tissues, and marrow, as these structures suffer most directly from the action of the x-ray. The results indicate also that one reason why the lymphocyte appears to be an important agent of defense in tuberculosis and other conditions may be its power to form antibodies.

In view of the destructive action of radium on the leukocytes and bloodmaking organs, it is altogether likely that it will be found to have the same general effect on the production of antibodies as the x-ray.

THE ABSENCE OF DEMONSTRABLE SPECIFIC ANTIBODIES IN RABIES CAUSED BY FIXED VIRUS *

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Our inability to isolate, grow, and identify microscopically the causal agent in rabies has rendered the study of its biologic reactions doubly difficult. A great number of organisms have been described. (Noguchi¹ has recently described an organism with which he has been able to produce rabies in animals.) Despite the fact that in the prevalent prophylactic treatment in rabies we are able to attain an immunity, or better a resistance, of an exceedingly high degree, specific serum reactions, especially reactions of diagnostic value, are as yet unknown. The mechanism of the immunity and the factors which produce this immunity have not been definitely determined so as to be generally accepted.

Babes² asserted that he had produced immunity by inoculating dogs with normal brain tissue. Fermi³ and Repetto⁴ believed that animals treated in this way possessed a greater immunity than animals immunized with rabies brains. Fermi⁵ found further that fresh egg-yolks and mixtures of cholesterol and lecithin were capable of lending animals an immunity to rabies. That the increased resistance obtained in this way is non-specific must be concluded. These experiments, however, lay open to doubt the specific immunity as supposedly obtained in the routine Pasteur treatment or in any of its many modifications.

The passage of street virus through rabbits so reduces the virulence for man that this material when inoculated into the latter is readily destroyed, presumably before the virus reaches the central nervous system. It has been assumed that in this way the formation of specific antibodies is stimulated upon which the subsequent immunity is dependent. It would seem plausible that a specific rabicidal serum could be produced. Kraus, Keller, and Clairmont⁶ were able to prove that the serum of immunized animals was rabicidal whereas that of non-immunized animals was non-rabicidal. As early as 1889 Babes and Lepp⁷ demonstrated that the serum of an immune animal was capable of rendering rabies virus non-virulent when allowed to act upon the latter in vitro. Such serum

* Received for publication June 22, 1915.

1. Presse méd., 1913, 21, p. 729; Berl. klin. Wehnschr., 1913, 50, p. 1931.

2. Cited, Kolle and Wassermann, Handb. d. Pathogen. Mikroorganismen, 1913, 8, p. 902.

3. Centralbl. f. Bakteriöl., I, O., 1907, 44, p. 475.

4. Ibid., 1909, 51, p. 581.

5. Ibid., 1908, 48, p. 357.

6. Ztschr. f. Hyg. u. Infectious-krankh., 1902, 41, p. 486.

7. Ann. de l'Inst. Pasteur, 1889, 3, p. 384.

has not, however, been found to lend an unquestioned passive immunity to other animals. Centanni⁸ noted that in an animal in the process of active immunization the rabicidal power of its serum was greater before full immunity was established than it was in the same animal at a later date when full immunity had been produced. It would therefore appear that even in active immunity the individual's resistance is not dependent alone upon a single type of antibody present in its serum. It has been assumed that in this later stage a histogenetic immunity has been developed in those body cells peculiarly susceptible to the action of rabies virus. These findings, apparently paradoxical in themselves, would almost forecast the ultimate fruitlessness of an attempt to obtain a reliable, specific, diagnostic serum reaction in rabies. It is probably because of this that so little work has been done along this line. Frugoni and Gargiano,⁹ when immunizing animals to rabies, obtained reactions not unlike anaphylaxis. There is no definite evidence, however, indicative that the sensitization in these instances was specific for rabies virus, and the possibility of employing such a sensitized animal for the determination of the presence of rabies virus in suspected brain is hardly feasible.

Following the work of Bordet and Gengou on complement-binding reactions, it was hoped that a method applicable to the diagnosis of rabies had been found. The essentials of a well-adapted antigen-antibody system were thought to be present; that is, a strong antigen in the powerful rabies virus and an immune serum. Heller and Tomarkin¹⁰ and Friedberger¹¹ were able to obtain an inhibition in hemolysis with this reaction when using extracts of rabies brain and serum from immunized animals. The reaction, however, was found not to be specific, inasmuch as non-virus-containing brain extracts employed as antigen also were bound to complement by immune serum. On the other hand, Baroni, Cinca, Ionescu-Mihaiesi,¹² Donati and Satta,¹³ and Berry and Mann¹⁴ found no evidence of antibody formation in rabies immune serum by complement-fixation tests. Dobrowolskaja¹⁵ got a definite inhibition in hemolysis in his experiments but found that sera from non-immune animals which were bled at the height of digestion were likewise capable of binding complement with his antigens. Bertarelli,¹⁶ who immunized rabbits to fixed virus, asserted that he had obtained a specific complement-binding which was not obtained in his control experiments. The direct variance in the results of these different investigators suggested the possibility that some of the non-specific reactions were due in part, not to an antibody for rabies virus, but rather to an antibody for the brain tissue with which the animals were injected at the time of immunization because of our inability to separate the brain tissue from its contained virus. With this in mind, Nedrigailoff and Sawtschenko¹⁷ prepared antigen from salivary glands of street virus animals and affirmed that they had obtained specific complement fixation with immune serum.

In our present method of immunizing animals, so much brain tissue is injected that apparently the overproduction of antibrain-tissue bodies

8. Deutsch. med. Wchnschr., 1893, 19, p. 1061.

9. Berl. klin. Wchnschr., 1911, 37, p. 254.

10. Deutsch. med. Wchnschr., 1907, 33, p. 795.

11. Wien. klin. Wchnschr., 1907, 20, p. 879.

12. Compt. rend. Soc. de biol., 1908, 65, p. 96.

13. Pathologica, 1908, 1, p. 96.

14. Jour. Exper. Med., 1910, 12, p. 339.

15. Centralbl. f. Bakteriöl., I, O., 1910, 56, p. 177.

16. Cited, Kolle and Wassermann, Handb. d. pathogen. Mikroorganismen, 1913, 8, p. 927.

17. Ztschr. f. Immunitätsforschung, 1911, 8, p. 353.

completely masks any specific antirabies-virus bodies present. With this in mind, I determined to ascertain whether or not any specific antistances could be found in the serum of animals inoculated with a single lethal dose of fixed virus. Here, the amount of brain tissue injected would be at a minimum, whereas the virus, which it must be assumed multiplies, would at some time prior to death have attained a relative maximum. True, the course in rabies caused by fixed virus in rabbits is so short and rabbits appear so susceptible that the outlook for obtaining either large numbers, or an early production of demonstrable antibodies did not seem encouraging. However, in some infectious diseases the production of specific antistances has been known to occur very early in the disease; thus, in typhoid fever, precipitins have been found even before agglutinins, which usually are found at the end of the first week.

I am indebted for my initial virus to Dr. A. Lagorio. This virus when inoculated subdurally produced the classical fixed virus paralysis almost invariably at the end of the fifth day. From the fifth to the seventh day, the rabbit frequently suffered repeated clonic convulsions, usually ending in death at the end of the seventh, or in the early part of the eighth day. During the first five days, those rabbits the brains of which had not been greatly traumatized in the inoculation appeared quite normal. They ate almost continuously, and drank considerable water. The leukocyte count increased usually daily with considerable regularity until the fifth day, when counts of 20,000 to 22,000 were obtained. During the paralytic stages, the counts made from peripheral blood frequently dropped even as low as 8,000 or 5,000. Babes has held that the leukocytes are active either directly or indirectly in combating rabies, and, inasmuch as antibody formation has been demonstrated by Hektoen¹⁸ and others to decrease in experimentally produced leukopenia, it was decided to make successive daily tests of the sera, inasmuch as the leukocyte counts varied in a very definite curve during the course of the disease. It was determined first to learn whether any ferments identifiable by Abderhalden's dialyzing method were present at any time during the disease. Accordingly, a series of eight rabbits and controls were inoculated subdurally, the former with a minimal lethal dose of brain containing fixed virus and the latter with an equal amount of normal brain. Babes and Pitulescu¹⁹ have noted that sera of patients receiving Pas-

18. Tr. Chicago Path. Soc., 1915, 9.

19. Compt. rend. Soc. de biol., 1914, 76, p. 267.

teur treatment for rabies split brain tissue of normal and rabid rabbits equally well, according to Abderhalden's dialyzing methods, and that this serum has no splitting action on human brain tissue. Substrates were prepared, according to the method of Abderhalden for preparing placental tissue substrates,²⁰ from normal rabbit brain, rabbit brain containing fixed virus, normal rabbit parotid gland and from parotid glands of rabbits afflicted with fixed virus rabies. Because of the great abundance of lipoids in brain tissue, it was also decided to learn whether fixed virus serum was more active on the substrates mentioned minus their alcohol and alcohol-ether soluble substances.

The substrates were extracted with absolute alcohol and then with equal parts of alcohol and ether in an incubator for several days. (Previously, substrates had been boiled until they were free from water-soluble, ninhydrin-reacting substances.) About 1 gm. of the substrate was placed in a dialyzing sac and covered with 1.5 c.c. of serum. The sac and its contents were then placed in a receptacle containing 20 c.c. of distilled water and the water and the sac's contents covered with a layer of toluene and placed in an incubator for twenty-four hours. The dialyzing sacs were prepared from a thin 6 to 7 percent solution of celloidin in a mixture of equal parts of alcohol and ether. Only such celloidin was used as would permit a dialysis of a solution of silk peptone and would retain albumins, as evidenced by a failure of dialysis of ninhydrin-reacting substances from normal blood serum.

From a study of Table 1, it is seen that both the sera of the rabies animals and those of their controls are capable of splitting normal brain substrates as early as the second day; and that this splitting power is slightly greater in the rabies serum than in the normal serum. In most instances, somewhat before the onset of paralysis, about the fifth day of the disease, the splitting action of rabies serum is almost completely lost for all substrates. Another factor observed in Table 1 is that in almost all instances substrates of rabies brain are more easily split by both the normal serum and that of the rabid animal. The substrates of normal and rabies parotid tissue are occasionally acted upon by the rabid, as well as the control serum but only rarely is this true and then to a far less degree.

In all instances, the same substrates extracted previously with alcohol and ether are also attacked to a less degree. It would appear that this extraction breaks up some complex fat-protein molecules which in the former substrates are split by the serum with the liberation of their amino-acid constituents.

20. Abderhalden's *Handb. d. biochem. Arbeitsmethoden*, 1912, 6, p. 226.

TABLE 1

NINHYDRIN REACTION. PROTEOLYTIC POWER OF RABIES AND CONTROL SERA FOR SUBSTRATES OF NORMAL BRAIN, RABIES BRAIN, NORMAL PAROTID, AND RABIES PAROTID

Duration, in Days, of Rabies and Controls, and Dialysis of Serum Alone	Normal Serum on Normal Brain Substrate	Rabies Serum on Normal Brain Substrate	Normal Serum on Rabies Brain Substrate	Rabies Serum on Rabies Brain Substrate	Normal Serum on Normal Parotid Substrate	Rabies Serum on Normal Parotid Substrate	Normal Serum on Rabies Parotid Substrate	Rabies Serum on Rabies Parotid Substrate
1-N — R —	— ...	— X—	— ...	+ X—	— ...	+ ...	— ...	+ ...
2 —	+	+	+	+	+	+	+	+
3-N — R —	— X—	+ X—	+ X—	+ X+	— ...	— ...	— ...	— ...
4-N — R —	+ X+	+ X+	+ X+	+ ...	+ X+	— X—	+ X+	— X—
5-N — R —	+ X—	— X—	— X+	— X—	— X—	— X—	— X—	— X—
6-N — R —	— X—	— X—	+ X—	+ X+	— X—	— X+	— X—	— X+
7-N — R —	+ X—	— X—	+ X+	+ X—	— X—	— X—	+ X—	— X—
8-N — R —	— X—	— X—	+ X+	+ X+	— X—	— X—	+ X+	+ X—

X = action on same substrate minus its alcohol and ether extracts.

N = normal serum.

R = rabies serum.

It was next determined to attempt to adapt the miostagmin reaction of Ascoli²¹ to the identification of any reaction between antigen and antibody in rabies caused by fixed virus.

A series of 21 rabbits was inoculated subdurally with brain containing fixed virus, and controls with normal rabbit brain tissue, as in the previous series. (The sera of 8 of these rabbits were sera from the previous series.) Of this series, 8 were bled, one each on successive days following the inoculation, 4 on the fifth day just previous to the onset of paralysis and at a time when their leukocyte counts were highest, 4 on the sixth day, 7 on the seventh day, and 2 on the eighth day. Test and control antigens had been previously prepared by grinding separately rabies brain, normal rabbit brain, and parotid gland from normal and rabid rabbits. These were desiccated rapidly at 37 C. and then extracted with absolute methyl alcohol in the proportion of 1:4 at 37 C. with frequent shaking for seventy-two hours. These extracts while still warm were filtered through Scheicher and Schüll filters, No. 590, and after cooling refiltered. The extracts in various dilutions were titrated with normal rabbit sera (diluted 1:20) until a mixture was obtained that would not cause a decrease in surface tension measurable by more than one drop as determined by a Traube's stalagmometer.²² It was found possible to use dilutions of antigen as concentrated as 1:20. The diluted serum and diluted antigen were mixed in the proportion of 9:1 and the reaction completed by keeping the mixtures at 50 C. in a water bath for one hour. The surface tension was measured by a Traube's stalagmometer by determining the difference in the number of drops for a definite volume of the serum-antigen mixtures after and before heating. The graduations on my stalagmometer are such that one drop of the serum dilutions, in general, averages 12 to 13 divisions of the scale. In this way, serum from animals at different periods in the course of rabies and from like controls were tested with extracts of normal and rabid brains and with extracts of parotid glands from normal and rabid animals (fixed virus) as seen in Table 2.

Ascoli, in applying this reaction in the diagnosis of various conditions as typhoid, carcinoma, etc., considers only such reactions positive as show an increase of two drops or more in the end reaction. From Table 2, it is seen that the controls in no instance reacted positively according to Ascoli's standard. Of the twenty-one rabies sera, only three reacted positively with rabies brain antigen, and, of these three, one reacted more strongly with normal brain antigen. One rabies serum reacted positively with normal brain antigen and failed to produce a like reaction with rabies brain antigen. Of these positive reactions, one occurred on the first day, the others on the seventh or eighth day. Parotid gland antigens failed, in the few instances in which they were utilized, to result in positive reactions.

21. München. med. Wehnschr., 1910, 57, p. 403.

22. Ascoli u. Izar, München. med. Wehnschr., 1910, 57, p. 1170, p. 2129; Abderhalden's Handb. d. biochem. Arbeitsmethoden, 1912, 5, p. 1357.

TABLE 2
MIOTAGMIN REACTION

Duration of Rabies or Control in Days	Normal Serum and Normal Brain Antigen	Rabies Serum and Normal Brain Antigen	Normal Serum and Rabies Brain Antigen	Rabies Serum and Rabies Brain Antigen	Normal Serum and Normal Parotid Antigen	Rabies Serum and Normal Parotid Antigen	Normal Serum and Rabies Parotid Antigen	Rabies Serum and Rabies Parotid Antigen
1.....	+0 ⁰ gtt.	+1 ¹ gtt.	+1 ¹ gtt.	+2 ² gtt.
2.....	+0 ⁵ gtt.	? gtt.	+0 ² gtt.	+0 ⁸ gtt.
3.....	+0 ⁰ gtt.	+0 ² gtt.	+1 gtt.	+1 ² gtt.
4.....	-0 ⁰ gtt.	+0 ² gtt.	-0 ¹ gtt.	+0 ⁸ gtt.
5.....	+0 ⁵ gtt.	+0 ⁸ gtt.	+0 ² gtt.	+0 ⁴ gtt.
5.....	+0 ² gtt.	+0 ⁴ gtt.	+0 ⁴ gtt.	+0 ⁴ gtt.
5.....	+0 ² gtt.	+0 ² gtt.	+0 ⁴ gtt.	+0 ⁸ gtt.
5.....	+0 ² gtt.	+0 ¹ gtt.	+0 ⁴ gtt.	+0 ¹ gtt.
6.....	+1 gtt.	+0 ¹⁰ gtt.	+0 ⁸ gtt.	±0 gtt.
6.....	+1 gtt.	+0 ⁴ gtt.	+0 ⁸ gtt.	+0 ¹ gtt.
6.....	+1 gtt.	B.+0 ⁵ gtt.	B.+0 ² gtt.	B.+0 ² gtt.
6.....	+1 gtt.	B.+0 ⁵ gtt.	B.+0 ⁵ gtt.	B.+0 ⁸ gtt.
6.....	+1 gtt.	B.+0 ⁸ gtt.	B.+0 ⁸ gtt.	B.+0 ¹ gtt.
7.....	+0 ⁵ gtt.	B.+0 ⁷ gtt.	+0 ⁵ gtt.	B.+0 ⁸ gtt.	+0 ⁵ gtt. (C)	?	+0 ⁵ gtt. (C)	+0 ¹ gtt. (C)
7.....	-0 ⁰ gtt.	B.+0 ⁸ gtt.	-0 ¹ gtt.	+2 ² gtt.
7.....	+0 ⁴ gtt.	+0 ⁸ gtt.	+0 ² gtt.	+0 ⁴ gtt.	+0 ⁵ gtt.	+1 ⁴ gtt.	+0 ⁸ gtt.	+1 gtt.
7.....	+0 ⁴ gtt.	+2 ¹² gtt.	+0 ² gtt.	+1 gtt.	+0 ⁵ gtt.	+0 ⁸ gtt.	+0 ⁸ gtt.	+0 ⁷ gtt.
7.....	+0 ⁵ gtt.	+1 ⁴ gtt.	+0 ³ gtt.	+1 gtt.	(C).+0 ⁸ gtt.	(C)+0 ⁸ gtt.
7.....	B.+0 ¹ gtt.	B.+1 ⁸ gtt.	B.+0 ² gtt.	B.+1 ⁶ gtt.
7.....	+0 ² gtt.	+0 ² gtt.	+0 ⁷ gtt.	+0 ¹ gtt.
8.....	+0 ⁶ gtt.	+3 ⁰ gtt.	+0 ⁶ gtt.	+2 ⁸ gtt.
8.....	+0 ² gtt.	+0 ⁴ gtt.	+0 ⁸ gtt.	+0 ⁷ gtt.	+0 ¹ gtt.	+0 ² gtt.

+1 = increase of 1 drop; +1⁸ = increase 1 $\frac{1}{2}$ drops; +0⁸ = increase $\frac{3}{4}$ drops; etc. Antigens diluted 1:100, except B, 1:20 and C, 1:50.

Inasmuch as these methyl alcohol "antigens" were rich in lipoids, it was thought that possibly some evidence of fat-splitting might be obtained with the same serum-antigen mixtures used in the miostagmin reactions. Accordingly, 5 c.c. of each mixture were titrated with N/50 NaOH and a phenolphthalein indicator before and after heating. In not one instance was the acidity after heating greater to a degree significant of lipoid-splitting, as seen in Table 3. In practically all instances, the acidity after heating for an hour was less than that of the mixture before heating. This, no doubt, was due to the expulsion of contained CO₂ in the original mixtures. In two instances, in sera of the eight-day rabies, the acidity was slightly greater in solutions of rabies sera and rabies brain. These two single instances, however, are insufficient, as compared with the great number of negative results, to warrant any general deduction.

TABLE 3
SPLITTING POWER OF SERA ON ALCOHOL-ETHER EXTRACTS OF NORMAL BRAIN AND RABIES BRAIN. TITRATIONS MADE WITH N/50 NaOH

Duration, in Days, of Rabies or Controls	Normal Serum and Normal Brain	Rabies Serum and Normal Brain	Normal Serum and Rabies Brain	Rabies Serum and Rabies Brain
1	— 0.03 c.c.	+ — 0.00 c.c.	+ 0.01 c.c.	+ — 0.00 c.c.
2	— 0.01 c.c.	— 0.02 c.c.	— 0.01 c.c.	— 0.03 c.c.
3	— 0.02 c.c.	— 0.02 c.c.	— 0.05 c.c.	— 0.03 c.c.
4	+ — 0.00 c.c.	— 0.04 c.c.	— 0.01 c.c.	+ — 0.00 c.c.
5	— 0.05 c.c.	— 0.04 c.c.	+ — 0.00 c.c.	— 0.02 c.c.
6	+ — 0.00 c.c.	+ — 0.00 c.c.	— 0.03 c.c.	— 0.02 c.c.
7	— 0.03 c.c.	— 0.02 c.c.	— 0.02 c.c.	— 0.01 c.c.
8	— 0.10 c.c.	— 0.05 c.c.	— 0.10 c.c.	+ 0.05 c.c.
8	+ — 0.00 c.c.	— 0.02 c.c.	+ — 0.00 c.c.	+ 0.06 c.c.

Inasmuch as precipitins are occasionally found early in the course of some infectious diseases, it was determined to learn whether any specific precipitin formation could be detected in rabies caused by fixed virus.

Precipitogen solutions were attempted by making extracts of rabies brain (1:10) with 0.85 percent NaCl solution. The brain was ground in a mortar and allowed to remain a few hours at 37 C. and then placed at +2 C. for twenty-four hours, after which it was filtered by forced aspiration through a Gouch filter. Similarly, control solutions of normal brain were prepared. A series of six rabbits was inoculated subdurally with fixed virus and controls

with normal brain tissue. Three rabbits and their controls were bled at the end of the fifth day, at which in most cases their leukocyte counts ranged from 18,000 to 25,000; the white cell counts of the controls were normal. Two of the remaining series and their controls were bled at the end of the seventh, and the one remaining rabbit and control on the eighth day. The presumed precipitogen-containing extracts of both normal and rabies brain were then diluted 1:2 progressively in a series of twelve tubes—a procedure giving an ultimate dilution of 1:1248. To a tube containing 0.5 c.c. of each of these progressive dilutions were added 0.2 c.c. of serum. In this way, serum of normal rabbit was tested with extract of rabid, and extract of normal brain, respectively, and likewise sera of rabies animals at the fifth, seventh, and eighth days of the disease were tested with the similar dilutions of the same extracts for precipitins. The tubes were incubated at 37 C. for an hour and allowed to stand twenty-four hours.

In no one instance could any evidence of precipitin formation be found.

CONCLUSIONS

Protective proteolytic ferments are occasionally found at different periods of rabies caused by fixed virus. While these are at times greater for virus-containing brain tissue, they are in no sense specific for the contained virus alone. Apparently no specific splitting of virus protein can be demonstrated in substrates of the parotid gland in rabies caused by fixed virus. This may be due entirely to the absence of virus in the gland, due, in turn, to the acute duration of the disease, which does not permit the virus sufficient time to extend to the gland, as it does in street virus rabies.

Specific lipolytic ferments do not appear to be contained in the serum of rabies of fixed virus.

No evidence of antibody formation can be determined in the rabies of fixed virus by the miostagmin reaction.

There is no formation of specific precipitins in rabbits with acute rabies caused by fixed virus.

THE FATE OF FOREIGN ERYTHROCYTES INTRODUCED INTO THE BLOOD STREAM OF THE RABBIT *

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Altho it has been definitely established that the introduction of foreign red blood corpuscles into an animal produces profound physiologic changes, as evidenced by the appearance of specific antibodies in the body fluids, the actual fate of the cells thus introduced has not been determined. From the point of view of general biology, as well as from that of the particular field of immunity, however, the actual fate of cells so introduced appears to be of much importance in that the site and mode of the destruction of the foreign cells undoubtedly bear a direct relation to the site of antibody production, concerning which but few data have been secured.

It is the purpose of this paper to contribute evidence that foreign blood cells introduced into the circulating blood stream are engaged by and destroyed within certain fixed tissue cells of the liver and spleen. In this connection I wish to express my thanks to Professor P. Kyes for suggestions and criticism relative to this work.

Kyes¹ has recently shown, in a wide range of animals, that the physiologic destruction of the animal's own red blood corpuscles is accomplished by specialized, fixed tissue phagocytes, which he designates as hemophages. The hemophages are confined largely to the liver and spleen and are of the same type in both these organs. In different animal species, the blood destruction accomplished by the hemophages may be carried on predominately, or even exclusively, in the one organ or the other, the site of the destruction, however, being constant for a given species under normal conditions. Thus in birds, amphibia, and lower mammals, the hemophages are most active in the liver, whereas, in the rodents and other high mammals, the hemophage activity is most marked within the spleen. Kyes has further shown that the destruction of the red blood corpuscles by the hemophages under normal conditions is attended with a freeing of the

* Received for publication June 22, 1915.

1. Internat. Monatschr. f. Anat. u. Physiol., 1914, 31, p. 543.

iron of the contained hemoglobin to such an extent that it is available for Perl's Prussian blue reaction in fixed tissues and affords a histologic method for the sharp differentiation of all active hemophages.

This demonstration of an actual intracellular destruction of red blood corpuscles within specialized cells under normal conditions, suggested the possibility that the same mechanism might operate in the destruction of foreign corpuscles introduced into the blood stream, and, further, that proof of this operation might be found in the increased activity of hemophages stimulated by the presence of a large number of foreign corpuscles. This increased activity should appear in the increased number of hemophages containing red blood corpuscles and displaying an iron reaction by virtue of the end products of the intracellular hemoglobin digestion.

To test the possibility of obtaining such proof, I have injected a series of twenty-six rabbits with beef corpuscles and compared the resulting hemophage activity in these animals with that of normal rabbits. Rabbits were selected as recipients, since, as Kyes has shown, in these animals, in contrast to many others, the liver does not contain active hemophages under normal conditions, the destruction of erythrocytes being accomplished by the splenic hemophages. Because of the known action of hemophages in the liver of other species, however, and the action of these cells under certain pathologic conditions, it was considered possible that, under the demand for a great increase in the destruction of erythrocytes, the liver even in rabbits would act as a supplementary organ in this function, and such a participation would contrast sharply with the normal condition. The red blood corpuscles injected were in all instances serum-free, being prepared by multiple washings in the isotonic salt solution. The concentration of corpuscles in the suspension actually employed for injection was in all instances equivalent to that of the blood as drawn. The unit for each injection was 5 c.c. of such a suspension and the avenue of introduction was the lateral ear vein. In the preparation of the tissues for histologic study, the technic employed was the same as that given by Kyes. This is an application of Perl's test for iron to fixed tissues in combination with suitable counter-stains. In detail, the method is as follows:

Fix thin slices of tissue for eighteen to twenty-four hours in Müller's fluid plus 5 percent mercuric sublimate. Imbed in paraffin and section to 4 microns. Fix sections to slide and stain five to ten minutes with acid carmin. Wash, and transfer to equal parts of a 2 percent aqueous solution of potassium ferrocyanid and a 2 percent aqueous solution of hydrochloric acid, the combined

solution containing also 2 percent of sodium chlorid. Remove after three to ten minutes, wash in distilled water, and pass quickly through a 1.0 percent aqueous eosin solution. Dehydrate in alcohol, clear in xylol, and mount in Canada balsam.

With this method, there are constantly to be found in the spleen of normal rabbits a large number of hemophages which are characteristically differentiated by virtue of a content of Prussian blue, the presence of which results from a partial liberation of iron from ingested erythrocytes. In the liver, on the other hand, no such differentiation of hemophages occurs. The differentiated cells of the spleen are similar to those described by Kyes in detail in the spleen of pigeons. Within a majority of such cells are to be observed erythrocytes in various stages of digestion. The number of hemophages active in the spleen at a given time varies somewhat for different individuals, even under normal conditions. But this variation is not marked and the tissues from a series of six normal rabbits serve as a basis of comparison in the following observations and are designated as Group I.

The rabbits injected with foreign corpuscles fall into four additional groups, according to the amount of hemophage activity displayed in the spleen and liver. Two factors were varied in the experiments, namely, the number of injections given to a single animal and the length of the period between the last injection and the killing of the animal. The results of the experiments, in general terms, show that the injection of foreign red blood corpuscles does increase the activity of the hemophages markedly. This activity, moreover, varies directly with the number of erythrocytes injected and is displayed in an increase of the number of hemophages containing these cells and the products of their digestion. Altho the great increase in the hemophage activity is apparent in the spleen, the clearest evidence as to this increase is to be seen in the liver, where the experimentation results in the presence of very great numbers of active hemophages in contrast to the total absence of such cells under normal conditions. As a basis for the discussion of the results in detail, Table 1 is introduced.

This table displays the relative number of hemophages observed in the spleens and livers of the total series of thirty-two rabbits. The series is divided into five groups. The six rabbits of Group I were normal controls and serve as a basis of comparison. It is to be noticed that active hemophages were constantly present in the spleens, but never in the livers of these normal rabbits. A very few active hemophages occur in the bone marrow also, but inconstantly, and the number is but slightly, if at all, modified by the experiment described.

In all the animals of Group II, the only distinctive result was a decided increase in the number of active hemophages in the spleen. No change appeared in the liver.

TABLE 1

Rabbit	Number of Injections* and Time Elapsing After Last Before Rabbit was Killed	Presence of Active Hemophages	
		in Liver	in Spleen
Group I—Controls			
1	Normal	—	+
2	Normal	—	+
3	Normal	—	+
4	Normal	—	+
5	Normal	—	+ ²
6	Normal	—	+
Group II—One Injection			
1	Sixteen hours	—	+ ³
2	Twenty-four hours	—	+ ³
3	Twenty-four hours	—	+ ³
4	Ninety-six hours	—	+ ²
5	Ninety-six hours	—	+ ²
6	Ninety-six hours	—	+ ⁴
7	Ninety-six hours	—	+ ²
8	One hundred and twelve hours.....	—	+ ¹⁴
Group III—One injection			
1	Twenty-two hours	+	+
2	Ninety-six hours	+ ²	+ ⁸
3	Ninety-six hours	+	+ ¹⁰
4	Ninety-six hours	+	+ ⁶
Group IV—Three injections, successive days			
1	Twenty-four hours	+	+
2	Twenty-four hours	+ ²	+ ¹⁰
3	Twenty-four hours	+ ²	+ ⁹
4	Twenty-four hours	+ ²	+ ¹⁰
5	Twenty-four hours	+ ²	+ ⁸
6	Twenty-four hours	+ ²	+ ⁷
7	Twenty-four hours	+ ²	+ ⁵
8	Twenty-four hours	+ ⁴	+ ⁵
9	Forty-eight hours	+ ²	+ ⁸
10	Forty-eight hours	+ ³	+ ¹⁵
11	Forty-eight hours	+ ⁵	+ ¹⁰
12	Forty-eight hours	+ ²	+ ¹⁰
Group V			
1	Four injections, alternate days. Twenty-two hours	+ ²	+ ¹⁰
2	Eleven injections, alternate days. One hour	+ ⁵	+ ¹⁵

* Each injection was the introduction intravenously of 5 c.c. of a suspension of washed beef erythrocytes in a concentration equivalent to that of the blood as shed.

The results in Group III differ from those in Group II in that, in addition to a marked increase in the hemophages of the spleen, there appear also, for the first time, active hemophages in the liver. In the instance of Rabbit 1, the active hemophages were found in the liver twenty-two hours after a single injection, this being the earliest occurrence noted.

The twelve animals included in Group IV received, in contrast to those of the preceding two groups, a multiple number of injections, namely, three. Eight

of these animals were injected on successive days and killed twenty-four hours after the last injection. The other four animals were similarly injected but were killed only after a lapse of forty-eight hours. All these animals displayed a very distinct increase in the active hemophages in the spleen and the abnormal presence of large numbers of active hemophages in the liver. In general, the rabbits of this group showed a distinctly greater hemophage activity, both in the liver and in the spleen, than that presented by rabbits of Groups II and III, which received but a single injection of corpuscles.

The two rabbits of Group V received a greater number of injections than any of the other animals. The former received four, and the latter eleven injections. In both instances, there was an extensive increase in the number of the active hemophages in the spleen and the presence of great numbers of similar cells in the liver. The results in the bone marrow were not definite.

From these results it is seen that the injection of foreign red blood corpuscles profoundly affects the hemophage activity of the recipient. Not only are the injected cells eliminated by an increase of active hemophages in the spleen, but also by an active participation of hemophages in the liver, whereas no such participation occurs in this organ normally in this species. It is to be observed also that, within limits, the degree of hemophage activity varies with the extent of the injection of foreign cells.

With the determination of the phagocytic activity of hemophages in the spleen and liver in the destruction of foreign erythrocytes, recurs the question referred to at the commencement of this paper: Do the cells which destroy foreign erythrocytes participate in the formation of their antibodies? If so, the liver and the spleen in animals injected with erythrocytes might well be expected to show a high concentration of antibodies to those cells. In a subsequent paper, I shall show that this is the case.

The Journal of Infectious Diseases

PUBLISHED BY THE MEMORIAL INSTITUTE FOR INFECTIOUS DISEASES

VOL. 17

November, 1915

No. 3

A NEW MEMBER OF THE ACIDURIC GROUP OF BACILLI *

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A generally accepted characteristic of the bacilli grouped together as aciduric or acidophilic, such as the bacillus bulgaricus, the bacillus acidophilus, the Boas-Oppler bacillus and others, has been the absence of gas production when grown in sugar media. Recently one¹ of us noted, in the course of the routine isolation of aciduric bacilli from stools, that a very few produced gas. We further found that when human feces are planted into N/20 acetic acid glucose broth, in some instances there may be gas production without the presence of yeasts. Further investigation revealed the fact that the gas was formed by a bacillus of the same morphology, staining, and general biologic properties as the bacillus acidophilus. Since a careful search of the literature has not disclosed a description of a bacillus of this type, we have considered it a new species and have named it *Bacillus acidophil-aerogenes*.

To obtain some idea of the frequency with which this bacillus occurs in human feces, 38 specimens from 37 individuals were examined, of which the majority were from very young infants. The feces

* Received for publication June 18, 1915.

1. Rahe: Jour. Infect. Dis., 1914, 15, p. 141.

of 9 individuals yielded this bacillus, of which 7 were adults and 2 infants, one of the latter being 8 days old and the other 6 months. Of 24 very young infants, ranging in age up to 19 days, in only one instance was the bacillus acidophilus found in the stool. On the other hand, a systematic examination of a few adult stools revealed its presence with only one exception. It seems probable that it occurs in the intestinal tract of most individuals and usually in much smaller numbers than does the non-gas-producing bacillus acidophilus of Moro.

This same aciduric gas-producing bacillus was also isolated readily from the feces of the sheep and the hen, but not from the few fecal specimens examined from dog, monkey, rabbit, guinea-pig, white rat, white mouse, goose, and pigeon. It is our belief, however, that this bacillus is widely distributed in nature and that an intensive search would reveal it in the intestinal tracts of a wide variety of animals.

In the isolation of this bacillus from the feces, a small amount of the material is seeded into +5.0 acetic acid glucose broth in fermentation tubes and incubated from 2 to 3 days. The tubes containing this gas-producing aciduric bacillus are then readily recognized. If the gas is due to yeasts, the fact will be indicated by the characteristic pellicle formation. This preliminary culture is then streaked on glucose oleate agar and several colonies are seeded again into the acetic acid broth, incubated for 2 days, and transferred to glucose broth tubes in which the natural acidity conferred by the meat has not been modified.

Either type of colony described by Mereschowsky for the bacillus acidophilus group may be produced by the bacilli of this type. That is to say, certain strains of this bacillus, when plated out on glucose oleate agar, formed small, round, white, opaque colonies about 1.2 mm. in diameter (Type 1), while other strains formed colonies that are gray, semi-transparent, smaller than a pinhead, with short, tooth-shaped projections (Type 2). After a few generations on oleate agar, this bacillus was induced to grow on glycerin agar of about +1.0 reaction and also on sugar-free agar of the same degree of acidity. The growth on these media was discrete and generally invisible before the second day. The colonies resembled those of streptococcus.

Altho, as a rule, as seen in fecal smears and on first isolation, the bacillus acidophilus is a slenderer rod than the bacillus bulgaricus, it

exhibits a marked tendency to vary in morphology, and, after a few generations on artificial media, certain strains may assume a size and morphology which are identical with those of the *bacillus bulgaricus*. A typical strain of this gas-producing variety, grown 24 hours on unneutralized glucose broth, developed strongly gram-positive bacilli, in length from 1.50 to 11.50 microns and in width about 0.80 microns. Altho the greater number of the bacilli averaged about 5 microns in length, strings up to 40 microns were present in almost every field. With Loeffler's alkaline methylene blue, the bacilli stained evenly. On glycerin agar, the bacilli were thicker and longer than those grown in sugar broths or on oleate agar. Occasional Y-forms were to be seen, and the strings were frequent, long and curved.

These gas-producing aciduric bacilli grow vigorously in sugar broths of proper acidity and very characteristically. The type of the bacillus which does not produce gas tended to cloud the medium evenly and rather lightly, whereas the gas-producing type formed a growth showing a marked tendency to adhere to the bottom and the side of the tube; but, if the latter culture was shaken, the medium became as heavily clouded as in the case of the *bacillus bulgaricus*. Like the bacillus of Moro, this gas-producing type was not motile. As regards the rate of growth in fluid media, it was found that this gas-producing type multiplied much more rapidly than did the *bacillus bulgaricus* or the *bacillus acidophilus* (Moro). In unneutralized glucose broth, *B. acidophil-aerogenes* gave rise, 7 hours after seeding, to a distinct cloud of growth, whereas the control cultures of the *bacillus bulgaricus* and the *bacillus acidophilus* (Moro) showed no evidence of growth.

This bacillus was found to ferment actively, at 37 C., the following carbohydrates: maltose, saccharose, lactose, raffinose, and dextrose. For these tests, unneutralized, sugar-free broth was used as a base. No growth occurred in mannite or dextrin broths and none in any medium at room temperature. *B. acidophil-aerogenes* forms acid more actively on the average in sugar media than does the *bacillus bulgaricus* or the *bacillus acidophilus* (Moro). With 16 cultures of *B. acidophil-aerogenes*, grown for 3 days in unneutralized dextrose broth, the average percentage of normal acid was 10.0; whereas with 12 strains of the *bacillus bulgaricus*,¹ incubated for 5 days, it amounted to 5.61, for 21 strains of the *bacillus acidophilus* Group 2 (Rahe), to 5.66, and for 20 strains of the *bacillus acidophilus*, Group 3 (Rahe).

to 5.09 The acid production of *B. acidophil-aerogenes* in terms of lactic acid is detailed in the following table.

TABLE 1

ACID PRODUCED IN 2% GLUCOSE BROTH IN TERMS OF LACTIC ACID (INCUBATION 72 HOURS AT 37 C.)

Jam.0.945	Baby-31.125	C-50.720	Case-20.720
B-21.220	Baby-11.080	H0.945	C-20.810
Ca-10.810	Buck0.855	C-30.810	F1.170
Be-90.855	Ca-30.855	Ca-40.720	C-60.765

The essential characteristic distinguishing this bacillus from the other hitherto described aciduric bacilli is the production of gas. This was produced, almost without exception, in broth containing any one of the five sugars mentioned. The amount of gas produced varied greatly for the several strains tested and even from time to time for a single strain. No increase in the amount of gas produced followed any increase in the amount of carbohydrate above 1%. Frequently an almost complete resorption of the gas occurred. After 72 hours' incubation, the maximal amount formed with any strain was 60% and the minimal 2%. For 16 cultures, the average amount formed in dextrose was 23%. The greater part of this gas was always hydrogen, the formula varying from 4H/1 CO₂ to 6H/1 CO₂.

Curiously enough, altho these gas-producing aciduric bacilli split lactose actively with a large production of acid, they grow poorly or not at all in milk. Only 1 strain clotted milk as early as 72 hours, while with 13 strains even partial clotting did not occur for from 15 to 20 days. Six cultures had no apparent effect upon the milk medium. The clot, when formed, was soft, with only a slight separation of whey. There were no gas streaks, even on continued incubation. In an effort to adapt these bacilli to a milk medium, several strains were replanted in this medium at 48-hour intervals for 5 weeks, but with no shortening in the time required to clot.

As is well known, the members of the aciduric group soon die out if kept at incubator temperature, on account of the increasing concentration of acid. In a comparative test, it was determined that a strain of the bacillus bulgaricus survived 6 days at 37 C. when growing in the glucose broth medium, while the bacillus acidophilus (Moro) lived for 15 days and *B. acidophil-aerogenes* for 8 days. At room temperature, however, *B. acidophil-aerogenes* survived much longer than did the other two types. At the end of 37 days, *B. acidophil-aerogenes*

was still alive, whereas the bacillus acidophilus (Moro) had died out after 15 days and the bacillus bulgaricus after 6 days. At ice-box temperature, all the cultures were alive at the end of 50 days, altho the viable bacilli had decreased greatly. In the last two tests, the cultures were grown for 48 hours at 37 C., and then placed in the dark at the respective temperatures.

We have found that an agglutinating serum for the bacillus acidophilus may be produced without difficulty. Rabbits were inoculated intravenously with the growth from glycerin agar cultures. After 3 or 4 injections a titer as high as 1:800 was obtained. Of 14 strains of *B. acidophil-aerogenes* tested against a serum immune to one member of this gas-producing group, 10 gave positive agglutinations. On the other hand, none of several strains of the bacillus acidophilus of Moro reacted to this serum. Contrariwise, a serum immune to a strain of the bacillus acidophilus of Moro did not clump any of a number of strains of the gas-producing type. Further experiments of this character seemed to indicate that the gas-producing group of aciduric bacilli exhibited a higher degree of homogeneity than did the several strains conforming to the type of the bacillus acidophilus of Moro.

OBSERVATIONS ON THE PROTEOLYTIC ENZYME OF *BACILLUS PROTEUS* *

STUDIES IN BACTERIAL METABOLISM, XL

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The rôle of enzymes in the manifestations of cellular activity, both in unicellular and multicellular organisms, has become a prominent subject of investigation. Evidence is accumulating which strongly supports the view that cellular function is intimately associated with enzyme action. The problem is inherently a complex one, but certain aspects of it may be advantageously approached through the bacteria. Here the enzymes of a single type of cell may be examined under varied conditions, quite free from complications introduced by heterologous cells. A study of bacterial enzymes, furthermore, should eventually lead to facts of practical importance, for enzymes probably play a not unimportant part in the phenomena of microbic disease.

One of the most important phases of cellular activity is that of nutrition; so prominent is the nutritional phase in the bacteria that it largely overshadows their other activities, and the nature and extent of their metabolism largely determine their function in the economy of Nature.

Bacterial enzymes are conveniently divided into two classes: those which are soluble—exo-enzymes—and those which are insoluble, appearing in solution when the bacterial cell is ruptured.

Among the soluble or exo-enzymes, the proteolytic enzyme produced by *Bacillus proteus* is noteworthy both for its activity and the readiness with which it may be obtained in solution free from bacteria. This enzyme is formed in plain broth and plain gelatin during the growth of the organism. It may be obtained in an active state, free from bacteria, by passing the broth or liquefied gelatin culture through a Berkefeld filter; the enzyme, being in solution, passes through with the filtrate, leaving the bacteria behind. Exposure of cultures of *B. proteus* to moderate heat, or to the germicidal action of 0.5% carbolic acid, kills the organism but leaves the enzyme practically unimpaired in activity.

* Received for publication June 30, 1915.

This enzyme and some of the conditions favoring its production have been studied by Auerbach,¹ Berghaus,² Kendall,³ Kendall, Day, and Walker,⁴ and others. The essential details may be summarized as follows:

1. The proteolytic enzyme of *B. proteus* appears in an active state in plain broth and plain gelatin cultures of this organism, from which it may be obtained free from organisms by filtration of the culture through unglazed porcelain.

2. The enzyme is not present in an active state in dextrose broth or dextrose gelatin cultures of *B. proteus*.

3. Very small amounts of dextrose added to plain broth or plain gelatin delay the appearance of this enzyme.

4. The enzyme, freed from bacteria, will liquefy sterile dextrose gelatin as readily as it will liquefy plain gelatin.

5. Neither moderate amounts of organic acids nor relatively large percentages of dextrose have appreciable influence upon the activity of the bacteria-free enzyme as measured by its gelatin-liquefying power in sterile gelatin.

One of us³ has advanced a theory in explanation of these various phenomena, which, in the specific case of *B. proteus*, rests upon well-defined features of its metabolism.

B. proteus, growing in nutrient broth or gelatin containing no utilizable carbohydrates (or other non-nitrogenous substances of similar composition), obtains its food requirements both for structure and energy from the protein constituents of the medium. In similar media containing utilizable carbohydrates as well, the structural needs are, as before, largely obtained from nitrogenous compounds, but the energy requirements are obtained at the expense of the carbohydrate. The proteolytic enzyme cannot be detected in those cultures so long as there is residual sugar. Small amounts of sugar delay the appearance of the enzyme. The production of enzyme in demonstrable form, under these conditions, is invariably associated with a relatively rapid increase in ammonia (deamination). Deamination is an index of protein decomposition. This soluble proteolytic enzyme therefore appears to be analogous to the soluble exo-enzymes of the intestinal tract, in so far as it prepares protein for assimilation by the organism.

1. Arch. f. Hyg., 1897, 31, p. 311.

2. Ibid., 1906, 64, p. 1.

3. Boston Med. and Surg. Jour., 1913, 168, p. 825.

4. Jour. Am. Chem. Soc., 1914, 36, p. 1965.

In the intracellular utilization of the products of its activity, however, the enzyme has no part. It is a noteworthy fact that sterile solutions containing the enzyme liquefy sugar gelatin quite as rapidly and extensively as non-sugar-containing gelatin, indicating that utilizable sugars in themselves do not interfere with the activity of the mature enzyme, altho they create conditions unfavorable for its formation.

If utilizable carbohydrate is added to the medium in which *B. proteus* is to be cultivated, the bacteria theoretically may derive that portion of their nutritive requirement which is to be transferred into energy, either from the nitrogenous protein derivatives or from the non-nitrogenous carbohydrates. That portion of their nutritive requirements which is needed for structural purposes⁵ must be obtained chiefly from the nitrogenous elements of the media, because the carbohydrates do not contain nitrogen. As a matter of fact, *B. proteus* invariably obtains the energy requirements from carbohydrates, if such be present in the medium in available form, leaving the protein constituents practically intact. A proteolytic enzyme is not theoretically required for the utilization of carbohydrate and the observations of Auerbach and others, quoted, indicate that none is formed in media containing fermentable sugars.

The experiments here reported were designed to show the effect of dextrose, a readily fermentable sugar, upon the formation and activity of the proteolytic enzyme of *B. proteus*. For convenience, the order observed in the discussion of the enzyme of *B. proteus* outlined at the beginning of our paper, has been maintained. The details of the analytic methods employed have been discussed in previous communications,⁶ and will not be repeated here.

The organism was grown in plain gelatin, and in plain gelatin of identical composition re-inforced by the addition of 1% dextrose. To facilitate filtration through unglazed porcelain filters, 5% gelatin was used in the entire series of experiments. Ordinarily 10-12% gelatin is used in the preparation of nutrient gelatin.

Table 1 shows the essential analytic details in concrete form. It will be seen that the plain gelatin was completely liquefied within the first twenty-four hours' growth, the reaction became progressively

5. Kendall: Jour. Med. Research, 1911, 24, p. 411; 1912, 25, p. 117. Boston Med. and Surg. Jour., 1911, 164, p. 288; 1913, 168, p. 825.

Kendall and Farmer: Jour. Biol. Chem., 1912, 12, p. 215.

Kendall, Day, and Walker: Jour. Am. Chem. Soc., 1913, 35, p. 1201; 1914, 36, p. 1937 (for full details and analysis).

6. Jour. Am. Chem. Soc., 1913, 35, p. 1201.

TABLE 1
THE INFLUENCE OF DEXTROSE UPON THE PROTEOLYTIC ENZYME OF *BACILLUS PROTEUS*

Days	Plain Gelatin			One Percent Dextrose Gelatin			
	Reaction			Reaction			Ammonia mg. per 100 c.c. Gelatin
	Alizarin	Neutral Red	Phenol- phthalein	Alizarin	Neutral Red	Phenol- phthalein	
1	+0.70	+0.30	+0.50	+1.00	+1.90	+1.80	0.7
4	-0.80	-0.30	+0.90	+3.20	+3.00	+2.60	1.4
9	-1.90	-0.90	+0.10	+4.20	+5.80	+5.90	1.4
16	-7.80	-4.00	-2.10	+3.20	+3.60	+3.80	2.1

+ = acid; - = alkaline. Reaction expressed in c.c. of N/1 acid or alkali to neutralize 100 c.c. of culture above that of the controls.
 L = liquefied; S = solid. Ammonia expressed as milligrams N increase per 100 c.c. culture over that of the controls.

alkaline and the deaminization as measured by the increase in ammonia, progressed rapidly. The sterile Berkefeld filtrate of this culture liquefied rapidly, showing the presence of a soluble proteolytic enzyme. These data collectively indicate an active and progressive decomposition of the protein constituents of the medium.

No liquefaction of the dextrose gelatin occurred even after 16 days' incubation, altho there was an abundant growth of the organisms. The reaction became progressively acid and there was practically no increase in ammonia (deaminization),—facts indicating a minimal action upon the protein constituents of the medium, but a decided fermentation of the dextrose. The sterile Berkefeld filtrate of this dextrose gelatin culture was wholly without visible action upon gelatin. No evidence of a soluble proteolytic enzyme was obtained.

TABLE 2

THE INFLUENCE OF DEXTROSE IN PROGRESSIVE AMOUNTS ON THE DEVELOPMENT OF *BACILLUS PROTEUS* IN PLAIN GELATIN

Days	Sugar-free Gelatin		0.1% Dextrose Gelatin		0.2% Dextrose Gelatin	
	Reaction	Mg. NH ₃ per 100 c.c.	Reaction	Mg. NH ₃ per 100 c.c.	Reaction	Mg. NH ₃ per 100 c.c.
1	—0.20	4.9 L	+1.05	a 3.5 L	+1.40	0.7
2	—0.40	13.3 L	+0.75	a 8.4 L	+1.35	a 3.5 L
3	—0.50	18.9 L	+0.70	a 14.0 L	+1.00	a 7.7 L
4	—0.95	49.7 L	+0.60	a 17.5 L	+0.95	a 9.1 L
5	—1.00	59.5 L	—1.50	a 34.3 L	+0.65	a 11.2 L
10	—4.65	183.4 L	—1.60	a 78.4 L	+0.60	a 35.7 L
16	—2.90	130.9 L	—1.70	a 100.8 L	+0.30	a 104.3 L

L = gelatin liquefied; enzyme in liquefied gelatin; a = sugar completely removed.

This experiment shows clearly that the addition of dextrose to gelatin cultures of *B. proteus* causes a striking difference in the nature and extent of the decomposition of the protein constituents of the medium from that observed when dextrose is absent: The reaction becomes strongly acid when dextrose is present, formation of ammonia (deaminization) is minimal, and the dextrose culture does not become liquefied even after prolonged incubation; there is no evidence of a soluble proteolytic enzyme in the dextrose culture.

An important question presents itself: Does the absence of a soluble proteolytic enzyme in a 1% dextrose gelatin culture of *B. proteus* indicate an inhibition of the activity of the enzyme by the acid products of fermentation, or does the dextrose actually prevent the formation of the enzyme? If the latter possibility were the one realized,

it would provide experimental evidence of a direct relation between enzyme formation and nutritional stimuli.

One method of obtaining information upon this somewhat complex problem is to add gradually increasing amounts of dextrose to plain gelatin and study the development of the organism with respect to metabolism and enzyme formation in their relation to this sugar. Table 2 summarizes such an experiment. Several important facts are clearly set forth therein:

1. In plain gelatin the evidences of proteolytic activity are unmistakable. The reaction, even in 24 hours, is alkaline; some deaminization has taken place; and the medium is completely liquefied. A sterile Berkefeld filtrate of this sugar-free culture will liquefy gelatin, showing that *B. proteus* has formed a soluble proteolytic enzyme in the medium.

TABLE 2—Continued
THE INFLUENCE OF DEXTROSE IN PROGRESSIVE AMOUNTS ON THE DEVELOPMENT OF *BACILLUS PROTEUS* IN PLAIN GELATIN

0.3% Dextrose Gelatin		0.4% Dextrose Gelatin		0.5% Dextrose Gelatin		1.0% Dextrose Gelatin	
Reaction	Mg. NH_3 per 100 c.c.	Reaction	Mg. NH_3 per 100 c.c.	Reaction	Mg. NH_3 per 100 c.c.	Reaction	Mg. NH_3 per 100 c.c.
+2.10	1.4	+2.75	0	+2.70	0.7	+2.80	0
+1.60	2.8	+2.30	0.7	+3.10	0.7	+3.10	0.7
+1.50	a 4.9 L	+2.25	2.1	+3.40	1.4	+3.45	1.4
+1.50	a 6.3 L	+2.20	2.1	+3.60	1.4	+3.85	1.4
+1.30	a 6.3 L	+2.40	2.1	+3.20	1.4	+3.35	1.4
+1.30	a 16.8 L	+2.45	2.8	+3.00	2.1	+3.45	1.4
+1.60	a 25.2 L	+2.35	4.9	+3.00	3.5	+3.30	3.5

L = gelatin liquefied; enzyme in liquefied gelatin; a = sugar completely removed.

2. The addition of 0.1% dextrose to the gelatin prior to inoculation fails to prevent the formation of the proteolytic enzyme. During the first 24 hours' growth the organism completely uses up the dextrose and makes some inroad upon the protein constituents of the medium. The only residual evidence of the dextrose is the acid reaction (+1.05) and a somewhat smaller amount of ammonia in the medium. The gelatin is completely liquefied and a sterile filtrate of this culture contains the enzyme in an active state.

3. The addition of 0.2% dextrose definitely delays the appearance of the proteolytic enzyme; at the end of 24 hours' growth some dextrose is still present in the gelatin; the effect of utilization of the dextrose upon the metabolism of the organism is clearly shown. The reaction is acid (+1.40), and there is no evidence of deaminization. The medium does not liquefy. At the end of 48 hours' incubation the dextrose is found to be completely used up; there is evidence of proteolytic activity at this time, shown not only by the complete liquefaction of the medium, but by a sudden increase in the formation of ammonia as well. In spite of the acid reaction of the medium, there appears to be no delay in the development of the proteolytic enzyme as soon as the dextrose is exhausted.

4. The addition of 0.3% dextrose to the gelatin causes a greater delay in proteolysis. Not until the third day of growth does the dextrose finally disappear. The effect of the dextrose upon the metabolism of the organism during this interval is very definite. As soon as the dextrose is exhausted, formation of ammonia begins immediately, and in spite of the very considerable degree of acidity the liquefaction of the medium is complete within a very few hours. A sterile Berkefeld filtrate of the culture on the third day liquefies gelatin energetically.

5. The addition of 0.4% dextrose or more, definitely prevents proteolysis. The reaction remains acid and even prolonged incubation of the cultures results neither in liquefaction nor increase in ammonia. The accumulation of acid products kills the bacteria.

This experiment shows that small amounts of dextrose, up to a maximum of 0.3%, progressively delay proteolysis and the appearance of the soluble proteolytic enzyme. Both proteolysis and the appearance of the enzyme follow immediately after the dextrose is exhausted. A considerable degree of acidity, the result of the fermentation of the dextrose, appears to be without noteworthy influence upon the production and rate of action of the enzyme.

DISCUSSION

The experiments show that 0.1, 0.2 and 0.3% dextrose delay but do not prevent liquefaction of gelatin by *B. proteus*. The reaction becomes acid during the exhaustion of the sugar, and the formation of ammonia (deamination) is restricted until the carbohydrate has disappeared. The reaction then becomes progressively less acid, formation of ammonia increases steadily, and the medium is rapidly liquefied. This experiment also furnishes evidence of the influence of utilizable carbohydrate upon the formation of the enzyme. It shows clearly that the formation of acid, which is produced incidental to the fermentation of the dextrose, is not in itself the decisive initial factor in preventing the appearance of the enzyme in an active state, because the enzyme may be demonstrated in the 3-day culture in 0.3% dextrose gelatin which is markedly acid in reaction (1.5%); at this time the organism has used up the dextrose and has attacked the protein constituents of the medium. This is indicated by an abrupt increase in the formation of ammonia. *B. proteus* appears to be unable, under the conditions imposed by this experiment, completely to use up 0.4% dextrose; there is no increase in the formation of ammonia and there is no evidence of proteolytic activity.

SUMMARY

In sugar-free gelatin, proteolysis and enzyme formation proceed rapidly from the start and the reaction becomes progressively alkaline as a result of the accumulation of the basic products of putrefaction. The addition of small amounts of dextrose—up to 0.3%—progressively retards the appearance of the enzyme until the sugar is used up. During this period acid products of fermentation, indicating an energetic action upon dextrose, accumulate rapidly, showing that the organism is attacking the carbohydrate, not the protein. When the sugar is exhausted, the organism is forced to derive its energy from protein constituents, and the enzyme is then formed to bring about the necessary changes in the protein to make it assimilable. Larger amounts of dextrose than 0.3% cannot be completely used up by *B. proteus* and no proteolytic enzyme is formed under these conditions.

These observations have shown that a soluble proteolytic enzyme is not demonstrable in dextrose gelatin cultures of *B. proteus* as long as dextrose is present. The enzyme promptly appears in an active state, however, as soon as dextrose can no longer be detected, notwithstanding the very considerable degree of acidity of the medium.

It might be assumed that the enzyme was present, but inactivated by the dextrose in itself and that the exhaustion of the dextrose removed the inhibition, leaving the enzyme free to act. This is not the case; sterile filtrates of liquefied gelatin cultures will liquefy sterile 1% dextrose gelatin as rapidly and extensively as sterile dextrose-free gelatin.

It is apparent from the foregoing discussion that moderate amounts of organic acids, resulting from the fermentation of dextrose by *B. proteus*, do not prevent the formation of the soluble proteolytic enzyme in gelatin after the dextrose is exhausted; it is equally apparent that dextrose in itself does not prevent the liquefaction of sterile dextrose gelatin by the mature enzymes, yet dextrose in gelatin cultures of *B. proteus* prevents their liquefaction.

The theory which best explains these various phenomena may be stated thus: The addition of dextrose to gelatin cultures of *B. proteus* protects the protein constituents of the medium—the bacilli utilize the carbohydrate in preference to the protein for energy requirements. Important direct evidence in favor of this sparing action of dextrose for protein is afforded by the prompt appearance of the proteolytic enzyme, and other indications of proteolysis, when the amount of

dextrose does not exceed 0.3% in the experiments cited. *B. proteus* does not form its characteristic soluble proteolytic enzyme in the presence of dextrose, under the conditions cited.

The tables show a rough parallelism between the liquefaction of gelatin and an increase in the formation of ammonia when dextrose is not present. It is conceivable that the ammonia which accumulates as the organisms act upon the gelatin might be either a by-product of the activity of the enzyme itself—but this is rather improbable, because other bacteria, as *B. typhosus* and *B. coli*, form ammonia in sugar-free media but do not produce soluble proteolytic enzymes—or it may be that the formation of ammonia is independent of the action of the enzyme.

The latter possibility would be in accord with the theory that formation of ammonia in cultures of bacteria is not a manifestation of

TABLE 3
COMPARISON OF THE ENZYME ACTIVITIES IN GELATIN OF STERILE FILTRATES FROM CULTURES OF *B. PROTEUS* IN PLAIN AND DEXTROSE GELATIN

Days	1				2				3			
	a	b	c	d	a	b	c	d	a	b	c	d
Plain	L	11.2	11.9	L	L	11.9	11.9	L	L	12.6	12.6	L
0.1% dextrose.....	L	11.2	11.2	L	L	11.2	11.2	L	L	12.6	12.6	L
0.2% dextrose.....	S	11.2	11.2	S	L	11.2	11.2	S	L	12.6	11.9	L
0.3% dextrose.....	S	11.2	11.2	S	S	11.2	11.2	S	L	11.9	11.9	S
0.4% dextrose.....	S	11.2	11.2	S	S	11.2	11.2	S	S	11.2	11.2	S
0.5% dextrose.....	S	11.2	11.2	S	S	11.2	11.2	S	S	11.2	11.2	S
1.0% dextrose.....	S	11.2	11.2	S	S	11.2	11.2	S	S	11.2	10.5	S

Column a = physical state of culture, L signifying liquefaction, and S, no liquefaction.

Column b = ammonia expressed as milligrams per 100 c.c. in tube at start (control).

Column c = ammonia expressed as milligrams per 100 c.c. in tube after 5 days' incubation.

Column d = physical state of tube after 5 days' incubation.

the changes which take place in the protein constituents of the cultural media prior to their assimilation by micro-organisms; it is essentially an index of the intracellular deaminization of the assimilated protein derivatives, incidental to their transformation into energy chiefly. The extracellular changes which the protein constituents of the media undergo are largely of the nature of hydrolytic cleavages; little or no deaminization takes place during this phase of bacterial metabolism. This is shown qualitatively in the following experiment:

Nine-day cultures of *B. proteus* in plain gelatin (completely liquefied) and 1% dextrose gelatin (solidified promptly when cooled to 18 C.; no liquefaction), respectively, were filtered through Berkefeld filters to remove all bacteria. The

sterile filtrates were added to sterile plain and 1% dextrose gelatin in the proportion of 1 c.c. of filtrate to 10 c.c. of plain and dextrose gelatin, respectively. Suitable controls were made and the various preparations were incubated at 37 C. for 5 days. Sterility was maintained throughout the entire process.

1. Sterile filtrate of 9-day culture of *B. proteus*, 1 c.c.—Sterile plain gelatin 10 c.c.

Control: Ammonia at start 12.6 mg. The mixture of filtrate and gelatin solidified promptly at room temperature. After 5 days' incubation at 37 C., ammonia 12.6 mg., the mixture was completely liquefied.

2. Sterile filtrate as above, 1 c.c.—sterile dextrose gelatin, 10 c.c.

Control: Ammonia at start 12.6 mg. The mixture of filtrate and gelatin solidified promptly at room temperature. After 5 days' incubation at 37 C., ammonia 12.6 mg., the mixture was completely liquefied.

DISCUSSION

One cubic centimeter of a sterile filtrate of a plain gelatin culture of *B. proteus* contains a soluble proteolytic enzyme which will completely liquefy ten times the volume of plain or dextrose gelatin. Dextrose gelatin was

TABLE 3—Continued

COMPARISON OF THE ENZYME ACTIVITIES IN GELATIN OF STERILE FILTRATES FROM CULTURES OF *B. PROTEUS* IN PLAIN AND DEXTROSE GELATIN

4				5				10				16			
<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>	<i>a</i>	<i>b</i>	<i>c</i>	<i>d</i>
<i>L</i>	15.4	15.4	<i>L</i>	<i>L</i>	16.8	17.5	<i>L</i>	<i>L</i>	28.0	28.7	<i>L</i>	<i>L</i>	15.4	15.4	<i>L</i>
<i>L</i>	13.3	13.3	<i>L</i>	<i>L</i>	14.0	14.0	<i>L</i>	<i>L</i>	18.2	18.9	<i>L</i>	<i>L</i>	13.3	12.6	<i>L</i>
<i>L</i>	12.6	11.9	<i>L</i>	<i>L</i>	12.6	12.6	<i>L</i>	<i>L</i>	14.0	14.0	<i>L</i>	<i>L</i>	11.9	11.2	<i>L</i>
<i>L</i>	11.9	11.9	<i>L</i>	<i>L</i>	11.9	11.9	<i>L</i>	<i>L</i>	11.9	11.9	<i>L</i>	<i>L</i>	11.2	11.2	<i>L</i>
<i>S</i>	11.2	11.2	<i>S</i>	<i>S</i>	11.2	11.9	<i>S</i>	<i>S</i>	11.2	11.2	<i>S</i>	<i>S</i>	10.5	10.5	<i>S</i>
<i>S</i>	11.2	11.2	<i>S</i>	<i>S</i>	11.2	11.9	<i>S</i>	<i>S</i>	11.2	10.5	<i>S</i>	<i>S</i>	10.5	10.5	<i>S</i>
<i>S</i>	11.2	10.5	<i>S</i>	<i>S</i>	11.2	10.5	<i>S</i>	<i>S</i>	11.2	10.5	<i>S</i>	<i>S</i>	10.5	10.5	<i>S</i>

Column *a* = physical state of culture, *L* signifying liquefaction, and *S*, no liquefaction.

Column *b* = ammonia expressed as milligrams per 100 c.c. in tube at start (control).

Column *c* = ammonia expressed as milligrams per 100 c.c. in tube after 5 days' incubation.

Column *d* = physical state of tube after 5 days' incubation.

liquefied as extensively as plain gelatin, indicating that dextrose in itself had no appreciable effect upon the activity of the mature enzyme. No increase in ammonia was detectable in these solutions, a circumstance showing that the complete liquefaction of the gelatin by the enzyme in the absence of bacteria is accomplished without any indication of deamination.

A similar experiment was made, using the sterile filtrate of the dextrose-gelatin culture in place of the sterile filtrate from the plain gelatin culture. It will be remembered that the dextrose-gelatin culture was not liquefied and no active proteolytic enzyme was demonstrable in it. The results were wholly negative; no liquefaction of the gelatin occurred and no increase in ammonia was detected. The analytic details are omitted.

A more comprehensive experiment was made in precisely the same manner, with sterile filtrates from the cultures of *B. proteus* in plain, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, and 1% dextrose gelatin, shown in Table 2.

Table 3 shows the essential analytic details. Column *a* shows the condition of the filtered culture, *L* signifying liquefaction, *S* no liquefaction. Column *b* indicates the amount of ammonia in the mixture of 1 c.c. filtered culture and 10 c.c. sterile gelatin at the beginning of the experiment, and Column *c* indicates the amount of ammonia after 5 days' incubation at 37 C. Column *d* shows the condition of the preparation after 5 days' exposure to 37 C., *L* signifying permanent liquefaction, and *S*, solidification when cooled to room temperature.

The results are self-explanatory. The table shows clearly that liquefaction of gelatin by sterile solutions containing the soluble proteolytic enzyme of *B. proteus* is not associated with an increase of ammonia. This is interpreted as indication that hydrolytic cleavage of the protein, shown by the liquefaction of the gelatin, is independent of deamination. The effect of dextrose upon the formation of the proteolytic enzyme is shown in the cultures containing 0.2%, 0.3%, and more of the sugar. A 48-hour culture of the organism in 0.2% dextrose gelatin was liquefied, but the amount of enzyme in 1 c.c. of the filtrate was insufficient to liquefy 10 c.c. of the gelatin to which it was added. A 72-hour culture, however, contained sufficient enzyme to accomplish this purpose. A similar condition was observed in the 3-day culture in 0.3% dextrose gelatin. Here, again, the enzyme was insufficient in concentration to liquefy 10 c.c. of gelatin. The filtrate of the 4-day culture, however, induced complete liquefaction in ten times the volume of gelatin. With 0.4% dextrose or greater amounts the formation of the soluble proteolytic enzyme in cultures of the organism was definitely prevented.

CONCLUSIONS

The foregoing experiments appear to be sufficiently definite to justify the following statements regarding the formation and activity of the soluble proteolytic enzyme of *Bacillus proteus*.

Bacillus proteus forms a soluble proteolytic enzyme in plain broth and plain gelatin. The mature enzyme may be obtained in an active state free from bacteria by filtering the culture through sterile Berkefeld filters.

The enzyme appears to be a preparatory enzyme in the sense that it prepares protein for assimilation by the bacteria; it has no demonstrable rôle in the intracellular utilization of the protein by the bacteria.

The liquefaction of gelatin by the bacteria-free enzyme is not accomplished by the liberation of ammonia; deaminization is an independent phenomenon associated with the intracellular utilization of the products of proteolysis by the organisms themselves.

The proteolytic enzyme does not appear in an active state in media containing utilizable carbohydrate; it appears only when protein or protein derivatives are being utilized for energy by *B. proteus*.

Dextrose added to broth or gelatin cultures of *B. proteus* prevents the formation of the enzyme; under these conditions the enzyme would appear to be unnecessary for the metabolism of the bacteria, because they are acting chiefly upon the dextrose.

Small amounts of dextrose—up to 0.3% in the experiments cited—prevent the formation of the enzyme until the sugar is exhausted; then the bacteria must utilize protein constituents of the medium for energy and consequently enzyme formation begins promptly.

Larger amounts of dextrose than 0.3%, which cannot be wholly exhausted by the bacteria, permanently prevent the formation of the enzyme. It is probable that the accumulation of products of fermentation of the dextrose, principally organic acids, create environmental conditions which inhibit the metabolism of the organisms and eventually lead to their death.

Dextrose in itself does not materially inhibit the activity of the mature, bacteria-free enzyme; sterile dextrose gelatin is as rapidly and completely liquefied as plain gelatin.

The observations presented here do not preclude the possibility that conditions other than those obtaining in the experiments recorded may influence the formation of the enzyme.

AN ECONOMICAL INTRACUTANEOUS METHOD FOR TESTING THE VIRULENCE OF DIPHTHERIA BACILLI *

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The testing of the virulence of isolated strains of diphtheria and diphtheria-like bacilli is important in particular for the following reasons:

1. Four to eight percent of individuals in localities where diphtheria is endemic, are carriers of bacilli morphologically and culturally like the diphtheria bacillus, but non-virulent in 30-50% of cases. At the Willard Parker Hospital in New York, Wilcox and Taylor found that 4.5% of the cases admitted to the scarlet fever wards were bacillus carriers, and that of the isolated organisms only one half were virulent.

2. According to Neisser, individuals who become persistent carriers after an attack of diphtheria show only non-virulent forms in fully 20% of cases. The absence of virulence in the organisms isolated from these carriers is probably permanent, since in our opinion these bacilli are not derived from the virulent bacilli which excited the disease, but rather from bacilli which were originally non-virulent. Therefore, if this fact were established, such individuals could be discharged from quarantine.

Morphologically, the non-virulent strains cannot be separated from the virulent, and in their sugar reactions the two types are in a majority of instances similar. Hence, the animal test must be employed for final diagnosis in doubtful cases. The method thus far in vogue for testing the virulence of diphtheria bacilli has been to isolate the strain, grow it for 48 hours in ascitic broth (1 part ascitic fluid and 2 parts veal broth), and inject 1 c.c. of the broth culture subcutaneously into a guinea-pig. A control guinea-pig is injected with the same amount of the broth culture, and also a small quantity of antitoxin (0.5 c.c. of a 100 or 200 unit antitoxin, which cannot be used for other purposes). If the strain is a true diphtheria organism, the con-

* Received for publication June 30, 1915.

trol guinea-pig will live, while the test guinea-pig will die in from 2 to 3 days, showing the lesions typical of death from the effects of diphtheria toxin—subcutaneous edema, often extensive, at the site of injection, congested and hemorrhagic adrenals, fluid in the pleural cavities, and congestion of the lungs with areas of partial consolidation. This method is reliable, but an autopsy should be performed in all cases in order to exclude death from other causes.

Recently Neisser has suggested that the virulence of cultures be tested as follows:

One loopful of a 24-hour Loeffler slant of the organism is suspended in each of 1 c.c., 10 c.c., and 100 c.c. of physiologic salt solution, and 0.1 c.c. of each suspension is injected intracutaneously into the abdominal surface of a guinea-pig. As a control, some antitoxin containing 8 units per cubic centimeter is added to an equal volume of the heaviest suspension, and 0.1 c.c. of the mixture is injected intracutaneously into the same guinea-pig. True virulent diphtheria bacilli will cause a characteristic local inflammatory lesion, with superficial necrosis, in from 48 to 72 hours, the intensity of the reaction depending upon the number of injected organisms and their virulence. The skin at the site of the control injection should remain normal in appearance. No lesions are produced when the bacillus xerosis or the bacillus hoffmanni, or the non-virulent diphtheria-like bacillus, is injected.

This method is analogous to that of Römer for the determination of small amounts of diphtheria antitoxin in sera. This consists of the intracutaneous injection of varying mixtures of the unknown antitoxic serum and a standard toxin; a slight excess of toxin produces a local necrosis which is in every way similar to that produced by virulent diphtheria bacilli, while a neutral or over-neutralized mixture shows no effect on the tissues at the site of injection.

The method recommended by Neisser is fairly satisfactory, but, following his directions, we have occasionally noted that the direct addition of antitoxin to the bacteria in the control injection immunized the animals sufficiently to affect the test lesions to a considerable degree. If the amount of antitoxin added is diminished to avoid this general immunization, the local action of the bacteria is not completely inhibited, so that lesions are found in both test and control areas.

For this reason the following modification of Neisser's method, which has been found to be both reliable and economical, is suggested.

METHOD

Two guinea-pigs, weighing about 350 grams, are used for the testing of from 4 to 6 different strains. One pig as a control receives 0.5 c.c. of antitoxin (about 500 units per cubic centimeter) intracardially at the time of the

tests, or intraperitoneally 24 hours before. The intracardial injection is the better, as it produces a complete inhibition of the local action of virulent bacteria in the control injections. The hair on the abdominal surface of each pig is removed, either by the application of a paste made of barium hydro-sulphid, or preferably by pulling the hair out. This easily can be done, with less pain probably than is associated with the prolonged irritation following at times the application of the depilatory.



Figure 1



Figure 2

Fig. 1.—Test animal. This guinea-pig received intracutaneous injections, at points A, B, C, and D, of suspensions of 4 different strains of morphologically typical diphtheria-like bacilli. At A and B there are no lesions, an absence of virulence being thus indicated in these strains; at C and D there are distinct, circumscribed, indurated lesions with beginning superficial necrosis, showing that these two strains are virulent.

Fig. 2.—Control animal. This animal received 4 injections corresponding to those received by the guinea-pig in Figure 1, and, in addition, 0.5 c.c. of antitoxin intracardially. No lesions were produced at C and D; hence the specificity of the lesions shown in Figure 1 is proved.

For the bacterial emulsion, a fresh 24-hour growth from an ordinary Loeffler slant or a glucose ascitic agar slant of equal size, is suspended in 20 c.c. of normal salt solution. It is important that the growth be not more than 24 hours old, since many of the bacteria die if the culture is kept for 48 hours or longer in the thermostat. Ice-box preservation of grown cultures also kills many of the organisms. Loeffler slants are used similar to those furnished by the New York city department of health for the purpose of diagnosis in diphtheria. They should have a fairly uniform surface size and be sealed with paraffin to prevent drying of the medium. It is also important that the medium be not too acid, since excess of acid is apt to inhibit growth to a considerable degree.

Suspensions of the cultures to be tested having been prepared in the way described, 0.15 c.c. of each is injected intracutaneously into both guinea-pigs. The abdominal surface is divided into 4 or 6 areas, in accordance with the size of the guinea-pig, and the injections are made as far apart as possible in order to avoid a fusion of the lesions. Four strains can be tested out on a medium-sized guinea-pig, and 6 on a larger one, without the danger of overlapping. A 0.5 c.c. or 1 c.c. Record syringe with a very fine steel or platinum iridium needle is suitable for the injection. If the injections have been made properly, a circumscribed elevation appears, which persists for from 1 to 2 minutes.

The results of the tests are noted in 24, 48, and 72 hours. Virulent strains produce a definitely circumscribed, local inflammatory lesion, which shows a superficial necrosis in from 48 to 72 hours. In the control pig the skin remains normal, if the injections have been accurately carried out. With non-virulent strains, no lesion will be found in either control or test animal.

In this way 4 cultures can be tested on 2 animals, as compared with 8 animals necessary in the older way. The control animal may be used again within a week for another set of tests without a further injection of antitoxin. By the use of large guinea-pigs, on which 6 tests can be made, 10 or 12 can be saved, a considerable advantage when a large number of strains are to be tested.

In testing by this method 20 non-virulent and 40 virulent strains of diphtheria bacilli, we obtained results corresponding exactly with those of the usual and less economical subcutaneous test for virulence. This method is being used at present in the routine virulence testing at the research laboratory of the New York city department of health.

THE ETIOLOGY OF "SYMPTOMATIC ANTHRAX" IN SWINE *

"SPECIFIC GAS-PHLEGMON OF HOGS"

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INTRODUCTION

Symptomatic anthrax, or "blackleg," has been reported and described by Mareck,¹ Born,² Battistini,³ and Balas and Willenberg.⁴ Arloing, Cornevin and Thomas,⁵ Glässner,⁶ and Wulff,⁷ on the basis of transmission experiments with material from cattle, denied the existence of this disease in pigs, and questioned the inoculation experiments of Mareck. Recently, von Ratz⁸ succeeded in infecting hogs with the bacillus of symptomatic anthrax, thereby reopening the question as to whether blackleg, or a similar infection, appears spontaneously in hogs. As far as I have been able to survey the available

* Received for publication July 1, 1915.

1. Monatsh. f. prakt. Tierheilkunde, 1896, 7, p. 489; 1897, 8, p. 174.

2. Veterinarius, 1897.

3. Quoted by v. Ratz, Ztschr. f. Infektionskrankh. d. Haustiere, 1913, 14, p. 1.

4. Berl. tierärztl. Wehnschr., 1908, 24, p. 734.

5. Le charbon symptomatique du boeuf, 1887.

6. Krankheiten des Schweines, 1912, p. 16.

7. Deutsch. tierärztl. Wehnschr., 1912, 20, p. 689.

8. Ztschr. f. Infektionskrankh. d. Haustiere, 1913, 14, p. 1.

literature, no cases of symptomatic anthrax in hogs have been reported in the United States. The observations and etiologic studies presented in this report are parts of our investigations dealing with the sequels of hog-cholera immunization.

HISTORY OF THE MATERIAL USED FOR THE INVESTIGATIONS

On Aug. 28, 1912, Dr. E. C. Deubler brought to the laboratory of the Pennsylvania State Livestock Sanitary Board a piece of muscle removed from the neck of a hog which had died the previous day. Clinically, the hog had shown a large, edematous swelling extending along the neck, blue-purplish discoloration of the abdomen, high temperature, distress, and diarrhea. Inasmuch as hog-cholera had existed in the piggeries on the farm from which the hog came, and all of the pigs had been immunized passively against the disease, the death of the animal was very important from a sanitary viewpoint. With the exception of the muscle lesions, no pathologic processes could be found indicative of hog-cholera. The piece of muscle, size 8 by 6 cm., was red-brownish, streaked by deep, brownish areas, very dry, friable, and spongy; the muscle fibers were separated by gas bubbles. On section, a small amount of a serous-hemorrhagic fluid could be squeezed out. The material had the characteristic acid odor of blackleg. The muscle was very light, small pieces floating on water. The microscopic examination revealed gram-positive and gram-negative organisms, with and without spores, resembling in many respects *Bacillus chauvæi* (Strain 1).

Dr. Deubler was instructed to make an epidemiologic investigation, and to procure a sick or a dead hog, if possible. On Sept. 9, 1912, a dead hog was sent to the laboratory and a report also submitted by Dr. Deubler to the writer, which read in part as follows:

"The piggery is located in a low, swampy place, but is constructed with concrete, so that the pigs rarely come in contact with the ground. Sixty pigs are kept in this place; no new hogs have been introduced into the pens since June last, at the time when all the hogs had been immunized against hog-cholera. The application of the serum was made subcutaneously on the inside of the thigh. The animals are fed on garbage, and drink the spring water which is collected from the hill-side near the piggery. No cases of hog-cholera have developed for the last two months.

"About August 25 one hog died; the cadaver was not examined. Another hog died on August 27, and a muscle specimen was sent to the laboratory. A third hog died on September 4, and the hog sent for autopsy died on Sept. 8, 1912.

"There is one hog which had, as had all of the others that died, a swelling on the left shoulder and neck for a few days. At the date of inspection the animal appeared normal; no swelling or rise in temperature could be detected. Altogether, four hogs have died with symptoms most suggestive of hog-cholera."

The hog sent to the laboratory for autopsy was examined about 16 hours after death, on Sept. 9, 1912.

AUTOPSY

Sow, weighing 220 pounds; good condition of nutrition; rigor mortis on limbs still present. The cadaver is bloated, the abdomen distended by gas. Along the abdomen the skin is reddish-blue and discolored, the discoloration spreading diffusely into the neck. Along the neck, and particularly near the

head, there is a diffuse, edematous-gaseous swelling. No wounds or scars can be detected in this region. From the mouth a small amount of froth is discharged. The anus is prolapsed and deep-bluish.

From an incision along the neck, a small amount of reddish fluid escapes; the subcutis is filled with small and large gas bubbles. Near the atlas, several muscle-groups are dark-reddish; on incision they crackle, are spongy and friable. A peculiar acid odor is marked. The intermuscular connective tissue is moist, and in some places slimy and yellowish. The exudate is filled with numerous gas bubbles. The peripharyngeal connective tissue is infiltrated by a serous-hemorrhagic fluid rich in gas bubbles; the sternocephalic and longus capitis muscles are deep-brown and emphysematous. The blood vessels in this region contain dark, liquid blood with numerous gas bubbles. In the left parotid region, extending along the left side of the neck, the gas formation in the intermuscular tissue is extensive; on incision a frothy, serous liquid runs off. Several portions of the brachiocephalic muscle are dark and spongy. The left masseter muscle is moist and contains numerous blackish, friable, dry areas, with gas. All the portions of muscle removed have a characteristic acid odor and float on water. The retropharyngeal, submaxillary, and cervical, as well as the prescapular, lymph-nodes are enlarged, soft and juicy on section.

In the peritoneal cavity is a small amount of reddish fluid. The intestines are grayish, distended by gas. The omentum and mesentery show slight imbibition. The lymph-nodes are enlarged and soft; the structure is indistinct. The membrane of the intestinal tract is thickened in places and covered by stringy mucus. No ulcers or scars are found in the ileum or cecum. The stomach is in an advanced stage of autolysis. The liver is enlarged; the capsule, smooth; the parenchyma spongy, foamy, and light-grayish. Gas bubbles are noted beneath the capsule in small aggregations. The bile is stringy. The spleen is small, the capsule wrinkled, the pulp dry, the stroma decidedly visible. The kidneys and pelvic organs are apparently normal. The peritoneum is reddish and discolored in places.

The thoracic cavities contain about 10 c.c. of a light-reddish fluid. The lobes of both lungs are only slightly collapsed, moist, and, on section, a large amount of frothy fluid escapes. The lobes of the left lung are rich in blood, but uniformly elastic, and contain air. In the pericardiac sac are a few drops of reddish fluid. The heart is small and flabby; the ventricles contain poorly coagulated, dark blood, rich in gas bubbles; the valves are normal. The myocardium is soft and light-brownish and it crepitates on incision; there are a few gas bubbles between the muscle fibers; the intima of all the blood vessels is deep-reddish (imbibition). In the trachea is a considerable amount of blood-stained froth. The larynx is diffusely infiltrated; the tonsils are reddened and studded with gas bubbles; on incision there are numerous small plugs in the crypts. The mucous membrane of the pharynx and larynx is discolored bluish and covered with stringy mucus. In the mouth and nasal cavities is a considerable amount of blood-tinged mucus. All the mucous membranes are cyanosed.

Diagnosis.—Gas-phlegmon of pharyngeal region of the neck; acute serous pharyngitis and laryngitis; foamy liver; pulmonary edema.

BACTERIOLOGIC EXAMINATION (STRAIN 2)

Bacterioscopic Findings.—Bacilli are found in smears from the affected muscles, heart-blood, liver, tonsils, etc. The organisms resemble anthrax bacilli with rounded ends; several rods, however, are very long and are connected

in filaments. Some forms are whetstone- or lemon- or spindle-shaped, so-called "clostridium" forms; some are decidedly club-shaped. The clostridium forms and some small rods show endogenous spore formation; in the long rods the spores are in polar position. Some bacilli retain Gram's stain only in the form of granules. Some are entirely gram-negative (Fig. 1).

The bacilli in the edematous exudate are actively motile when slightly heated. The preparations from the depth of the muscle treated with Lugol's solution show that numerous organisms are brownish, either in stripes or in granules (so-called "granulose"). The findings in Specimen 1 correspond with those in Specimen 2.

The impression preparations from the surface of the liver show gram-positive rods in aggregations of long filaments and threads; spore-bearing forms are rare; gram-negative rods of the same size and thickness are numerous.

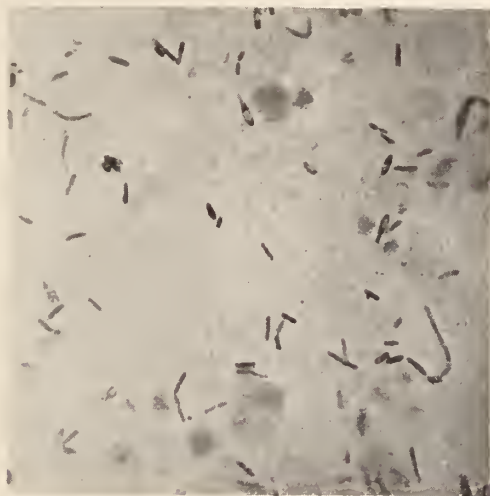


Fig. 1.—Smear from the diseased muscle (Strain 2). Carbolthionine. $\times 1000$.

The filaments of rods are frequently in thick clusters, in which the isolated threads run parallel. Similar smears are obtained from the peritoneal lining, the serosa of the intestines, the kidneys, and the spleen.

Cultural Results.—Aerobic cultures (Strains 1 and 2) from the depths of the affected muscle portions were all sterile. The heart-blood (Strain 2) contained aerobically an organism which was identified later as *B. coli*. From the spleen, liver, kidneys, and tonsils were isolated streptococci and *B. suispestifer*.

The anaerobic cultures prepared from the muscles, heart-blood, liver, and tonsils in glucose agar, brain medium, and blood broth developed freely a gram-positive, spore-bearing rod, but no isolated colonies could be detected. The brain tubes heated for 10 minutes at 60 C. gave, in 24 hours, with marked gas production, a pure culture of the organism described. The medium remained pinkish. Plate cultures, incubated under hydrogen in Novy jars, gave

unsatisfactory results. The culture medium was beef extract agar and gelatin. Later, however, with the use of brain infusion agar, good results were secured.

Animal Inoculations.—The dried (at 37 C.) and powdered muscle substance was suspended in salt solution and inoculated into guinea-pigs, rabbits, and pigeons. All the animals died in from 16 to 36 hours. The autopsies revealed pronounced serous-hemorrhagic subcutaneous edema, the organism being found in the fluid and in aggregations of filaments on the surface of the liver.

These findings (filaments brain medium unchanged), which will be more definitely described later, led to the conclusion that the hogs were not suffering from "blackleg," but from an unknown, closely related organism.

The two specimens collected from the hogs were identical in the muscle lesion. From the history, one can conclude that 5 hogs of the piggery suffered from the same infection, to which 4 succumbed. Unfortunately, only 2 specimens could be examined, and a post-mortem made on 1 only. The muscular lesions were doubtless of the greatest interest; one regrets, however, that the history does not state to what extent the process had existed before, and to what degree it developed after, death. That a post-mandibular swelling existed is stated in the history; whether the hogs suffered from anorexia could not be determined afterwards. The anatomic alterations, consisting in serous-hemorrhagic infiltration of the subcutis, and hemorrhagic myositis, were confined to the peripharyngeal regions and the adjacent muscles. The gas formation, however, extended all along the neck to the shoulder, and in regions where no muscular changes could be seen. Along the hind legs no subcutaneous gas formation was seen. The process was apparently confined to the neck and, even if gas production occurred after death, it remained confined to the primary focus of infection.

As the cause of the lesions described, a strictly anaerobic, spore-producing bacterium was recognized, which in many respects differs from the common animal pathogenic organisms. A detailed description of the bacterium will be given later. The organisms were present in large numbers and in pure condition.

The postmortem findings suggest as portal of entry the upper digestive or respiratory tract. It is highly probable that the tonsils served as direct portal of entry, as the organisms were found there in large numbers. The inflammatory process apparently radiated from these organs. The tonsils of hogs are known to be frequently the portal of entry for bacterial infection, as for instance, in anthrax,

tuberculosis, etc. The fact that the gas formation had already badly altered the architecture of the lymph-node makes it difficult to state whether or not the plugs found in the crypts harbored the organisms and caused the infection to take place at a time when the animal proved to be otherwise lowered in vitality. No wound or other epithelial defect was noted on the head or neck which could have served as portal of entry.

The evidence and information on hand do not permit any conclusion as to when the infection occurred and how long its incubation and duration were. Some transmission experiments, described later, throw some light on this question. The number of cases indicates that a common source of infection was probably responsible. However, not every animal was susceptible to the same degree. One animal is known to have recovered. It was quite natural that the owner should have accused the hog-cholera immunization of being responsible for the death of the animals. But this idea can easily be refuted in that the time interval between immunization and the death of the hog was about 2 months. Experiments, which will be discussed later, show that the incubation time is probably less than 3 days. Cases have been known to occur in which malignant edema has developed following the careless application of serum. Usually the morbid process starts and spreads from the seat of inoculation; in our cases, the serum was applied to the inner side of the thigh.

The relation of this disease to hog-cholera is of particular importance. The clinical symptoms—discoloration of the abdomen, diarrhea, general depression, and high temperature—suggest hog-cholera and, without postmortem examination, these cases would undoubtedly have been classified with this plague of swine. The false deductions which would result from mistakes of this kind—with regard to the value of immunization against hog-cholera, etc.—are so apparent from the detailed discussion of this epidemic that the matter requires no further emphasis.

On preliminary examination, the isolated organism showed so many differences from the generally poorly described and poorly studied animal pathogenic anaerobes, that a detailed study suggested itself. The splendid methods devised by v. Hibler⁹ for the study of anaerobes were followed in their essential points, particularly when details of

9. Untersuchungen über die pathogenen Anaeroben, etc., 1908.

importance concerning the points of differentiation between closely allied organisms had to be established. The results are reported in the following paragraphs.

BACTERIOLOGIC STUDY OF THE ISOLATED ANAEROBIC ORGANISM

The bacteria isolated from the lesions in the two hogs correspond in every respect; therefore, when not specially noted, the findings apply to both Strains 1 and 2.



Fig. 2.—Impression preparation from the liver surface of Rat 38 (young). Carbolthionine. $\times 1000$.

MORPHOLOGY

In general appearance the organism is a bacillus of varying size and shape. It is motile and spore-producing. The smallest rod is about 3 to 4 microns long, and 0.5 to 1.0 micron thick (unstained; Leitz's stepmicrometer); it is straight or slightly bent or curved. These forms appear in the serous exudate, in the muscle, and in the cultures. Fragments of such rods are frequently seen, resembling streptococci or other cocci-like aggregations. The longest rods

measure 13 to 15 microns, the thickness being about 0.8 micron; they are straight, bent, or wavy. The microphotograph (Fig. 2) of the organisms from the surface of the liver of a rat gives a good idea of these forms of the streptobacterium. The thickness of these filament-like rods is rarely even. In some cases they are very fine, and in others they show knob-like thickenings. The ends are mostly rounded. Some, however, show sharp-cut ends, like anthrax bacilli. The threads are sometimes segmented in small straight rods or are undivided and bent; the fine rods are rarely segmented. Some short rods also show club-shaped enlargements at one pole. By far the most interesting forms are the whetstone- or cigar-shaped, bloated or clostridium forms. They vary in size from 3 to 5 microns. Some are oval-shaped, some are pear-shaped, in most of the



Fig. 3.—Flagella stained by Zettnow's silver stain. Surface culture, 16 hours old, on glucose ascitic fluid agar (+ 0.5) in hydrogen vacuum. $\times 1000$.

cases containing elliptical spores. These elliptical spores are of varying sizes; they are mostly 2 to 5 microns long and 1 micron thick. They are in polar or central position and very refractile. The polar spores are very frequently seen at fine rods; the organisms appear drumstick-like. The central spores usually have a small amount of protoplasm attached at each pole. The free spores usually stain slightly in the center, so that the impression is given of a ring-like arrangement in the spore.

The non-sporulated rods in animal or cultural material are actively motile. By Zettnow's silver stain, numerous peritrichal flagella can be demonstrated. From glucose ascites fluid agar slants, organisms were obtained which had from 4 to 16 flagella (Fig. 3). The motility in cultures is exceedingly variable, apparently depending on numerous factors. In glucose broth or in glucose

agar, with the customary reaction, when 24 to 48 hours old, very few motile bacilli are seen; in ascitic fluid agar with a reaction of 0.5, most of the rods develop flagella and are motile. These observations confirm the findings of v. Hibler.¹⁰ They will be discussed more in detail later. The acid reaction, even when produced by the organisms through the fermentation of the carbohydrates, is apparently inhibitive of locomotion.

No giantwhips — as they have been described by Novy¹¹ for his organisms of malignant edema — could be demonstrated.

The bacilli stain readily with ordinary aniline dyes; preparations which are particularly clear are obtained with thionin. Most of the organisms stain uniformly, particularly those in the edematous fluid of guinea-pigs. In the muscular lesions of hogs, however, a large percentage of the bacteria stain faintly and irregularly. Gram's method gives positive results when 95% alcohol is used for decolorization; in all the preparations which are treated with acetone alcohol (1:3) mixtures, the organisms become rapidly and uniformly decolorized. In the preparations from cultures and animal material a certain percentage of bacilli always stain faintly gram-positive; some are gram-negative. The fine, filament-like rods are in most cases gram-negative. In old cultures the organisms are mostly gram-negative; some lemon-shaped forms retain the rosaniline dyes in the form of granules. The young, thick and short rods are always decidedly gram-positive.

Numerous tests to demonstrate the presence of "granulose" in the bacteria have always been successful. Dried and fixed muscle smears or preparations from glucose, starch, saccharose media, treated with weak Lugol's solution, show a large number of bacteria regularly staining brownish. Some show patchy-staining reaction; either the poles are reddish-brown or the bacillus is striped with two to three light-brownish bands. The clostridium forms frequently show marked granular "granulose-infiltration." The presence of "granulose" is constant in young cultures in the presence of carbohydrates. The extent, however, is frequently variable, and depends on factors which I have not as yet carefully studied. In any case, the preparations with Lugol's solution demonstrate clearly the described pleomorphism of the bacilli and, in our case, permitted an early diagnosis.

The various forms were distributed in the animal body and in cultures as follows: In the muscle of the hogs the short rods, the clostridium forms, and the spore-bearing rods were present in equal numbers and in regular distribution. On the surface of the liver and serous membranes of all animals examined, the long filaments of segmented or non-segmented bacilli in parallel aggregations, without spores, were the rule. In one instance, the filaments were so predominant in the peritoneal fluid that true clumps and coils of the rods were demonstrated (Fig. 2, the surface of the liver of Rat 38, Table 4). The uterine contents of Rabbit 5 were rich in whetstone- and lemon-shaped clostridium and spore-bearing forms, together with short rods. In brain and ascitic or serum media cultures the various forms, particularly spores, were always present and resembled the microscopic pictures of those made from animal material. In broth or agar cultures spores could rarely be detected; mostly medium-sized rods were present.

10. Untersuchungen über die pathogenen Anaeroben, etc., 1908, p. 145.

11. Ztschr. f. Hyg. u. Infektionskrankh., 1898, 27, p. 222 (Figs. 1, 2, 3, and 4).

CULTURAL CHARACTERISTICS

Preliminary tests had shown that the bacillus grows under anaerobic conditions. The usual media for anaerobes, as blood broth (Kitt) or brain decocotion (v. Hibler), gave a satisfactory growth without difficulty; but in some instances when the material was contaminated with aerobes (cocci, intestinal organisms) these media were not suitable for the biologic study of the bacillus. Pure cultures were obtained therefore by plating or deep tube methods. Most of the cultures were obtained by using material from inoculated guinea-pigs. The portions of muscle of the hogs were dried at 37 C. and kept sealed in tubes. Before inoculating the animals, small pieces were ground in a mortar to a fine powder and salt solution gradually added (about 10 c.c. to 1 gm. of substance). The suspension, heated for 30 minutes at 56-60 C., was subcutaneously introduced (on the back or in the knee-fold, by means of capillary pipet) into guinea-pigs (weight 300-400 gm.). Soon after the death of the animal the autopsy and bacteriologic examinations were carried out.

The plating method gave unsatisfactory results until I used brain or rabbit meat infusion agar containing only 0.5% glucose with the addition of sterile guinea-pig tissues. Smith,¹² Kitt,¹³ and Grassberger and Schattenfroh¹⁴ recommended, for the isolation of *Bacillus chauvæi*, the addition of sterile beef muscle. In the experiences of numerous workers, as well as my own, such material is rarely obtainable and even in the best organized laboratories is not always at hand. The liver, kidneys, and muscles of the guinea-pig offer a splendid substitute. Rabbit tissues are very unsatisfactory and more expensive than those of the guinea-pig when only a few pieces are necessary for the cultures. Also organs of cat and mice gave poor results. The tissues were chipped to small pieces in sterile Petri dishes and equally distributed over the bottom. The boiled and cooled agar (reaction +0.5), inoculated with some edema or abdominal fluid or heart-blood properly diluted, was poured into the plates and quickly solidified on an iced glass plate. The plates were placed in a Novy jar, which was freed from oxygen by the combined vacuum, hydrogen-pyrogallie acid method. The hydrogen was produced in a Kipp's generator and washed according to the method of Heim.¹⁵ In numerous tests, the recently described method of Heim,¹⁶ of combining the pyrogallie method of Lentz with hydrogen, gave perfect results so long as the proper agar and fresh tissues were used.

To obtain isolated colonies, in many instances the deep stab cultures in agar or gelatin proved valuable. The so-called "Burri-tubes" combined with use of sterile organs need only a simple equipment and are therefore more readily at hand than the plating methods; if long tubes are used, all three dilutions can be stratified in one tube. The third dilution is placed at the bottom of the tube, in close contact with a piece of guinea-pig liver, etc.; the second and first dilutions, in turn, are poured on top, and the contents immediately solidified in ice water. For gelatin cultures, only absolutely fresh preparations of a 10% concentration with a reaction of 0.4 were found reliable. Infusions of veal, brain, or rabbit flesh were used when not otherwise mentioned. Ascitic fluid was frequently added to the agar.

12. Centralbl. f. Bakteriöl., 1890, 7, p. 502.

13. Bakterienkunde und pathologische Mikroskopie, etc., 1908, p. 300.

14. Arch. f. Hyg., 1904, 48, p. 1.

15. Lehrbuch der Bakteriologie mit besonderer Berücksichtigung der Untersuchungsmethoden, Diagnostik und Immunitätslehre, 1911, p. 126.

16. Ztschr. f. d. ges. exper. Med., 1914, 3, p. 215

All the other media used for the differentiation were prepared and used as outlined by v. Hibler,¹⁷ Foth,¹⁸ and others. Liquid media in test-tubes were kept in Buchner's tubes or stratified with hydrocarbon oil. Flasks with broth or milk cultures were kept according to Wright's method or in Novy jars with hydrogen and vacuum. In some instances, also, brain media were inoculated, and from these, broth cultures obtained. Slant cultures were prepared on ascitic fluid agar (1% glucose) and sealed under hydrogen. On some occasions also gelatin agar (Kitt) was used for stab cultures.

Plate Cultures on Plain Agar.—By using the method of Foth, isolated colonies could be obtained on the plates. On opening the Novy jar a rancid odor escaped, resembling closely the odor of blackleg cultures. The agar was split by gas bubbles of various sizes; the water of condensation accumulated in these parts was turbid. Around the tissue pieces were seen diffusely spreading, grayish, irregular, fluffy colonies. The size of these colonies varied considerably. Some large colonies were round, smooth-edged, and finely granulated. Most of the colonies, however, were filamentous at sixty times' enlargement. From a distinctly yellowish center, filaments radiated regularly in the agar mass. When such colonies grew together, leaf-like structures could be observed. In the depth of the agar these fluffy colonies were more developed than on the surface. The outer zone of some of the colonies was more opaque than the center; the filaments were frequently toothed or club-shaped. Microscopically, motile, gram-positive rods were always found with very numerous spores, clostridium forms, and "granulose."

Streak Cultures on Agar (with Ascitic Fluid) in H Atmosphere.—A veil-like, grayish film or large, irregular colonies developed, resembling those of *B. proteus*. The growth was very fine and not characteristic.

Shake Cultures in 1% Glucose Agar.—In the first dilution there were abundant gas production and heavy growth, so that the water of condensation collecting between the agar fragments was very turbid and yellow-grayish. The odor was decidedly rancid. Properly isolated colonies were about the size of a large pin-head, spherical or disc-like; they were roundish, the edges very fluffy, wavy, and bush-like. When enlarged, the thick, filamentous, radiating structure of the colonies was marked.

Shake Cultures in Glucose (1%); Gelatin (12%).—In the first dilutions a uniform turbidity developed from the middle to the bottom of the tube; a few gas bubbles separated the medium; after 6 to 10 days' incubation at 22 C., the deeper layers of the medium were usually liquefied. Isolated colonies in properly prepared tubes were like cotton flakes; they were grayish, filamentous, and had irregular margins. Some were roundish with radiating inside structure and smooth margins. The liquefaction took place very slowly. The gas bubbles formed were small.

Stab Cultures in Agar and Gelatin (1% Glucose).—For only about 1.5 cm. below the surface of the medium bulk, was the growth to be noticed. It was not characteristic. The growth was filiform in agar; more rhizoid in gelatin, where liquefaction was also noted. The gas production took place very early in the growth and was extensive.

Cultures in Broth.—Only when sterile tissues had been added, was growth observed. In 24 hours, decided turbidity and pronounced gas production were noticed. Usually, in from 48 to 100 hours, the cloudiness had settled to the

17. Untersuchungen über die pathogenen Anaeroben, etc., 1908, pp. 83, 97.

18. Ztschr. f. Infektions-krankh. d. Haustiere, 1909, 6, pp. 230, 253.

bottom of the tube and there formed a thick, whitish sediment, varying in height from 0.3 to 3 cm., according to the age of the transplanted culture or to the previous animal passage. When shaken, the whitish deposit was easily suspended as granular material, which sedimented rapidly, however. These observations were particularly constant in test-tube cultures when large amounts of material were used; the growth continued for at least eight days, and the sedimentation was accordingly slow. The odor of the cultures was slightly rancid, never repulsive and of putrefactive character. The microscopic findings were interesting: Long, gram-positive, filamentous aggregations prevail in the first 16-20 hours; afterwards, short or abnormally shaped rods, whetstone-, cigar-, or pear-shaped forms with spores and "granulose," were very numerous. Sporulation and "granulose" production were well marked. In five-day-old cultures, mostly gram-negative rods and free spores, were found.

In Broth with 1% Glucose or Sodium Formate (0.3%).—Growth also took place in this medium in the absence of sterile tissues. The growth, the gas production, and the sediment were more pronounced and abundant. In Dunham's peptone solution, the growth was poor, or very slight; gas production was present.

In Glucose (1%) Sheep- or Horse-Blood Broth (Kitt).—In this excellent medium, the organisms grew very readily under aerobic conditions. After 34 hours a heavy froth arose on the fluid bulk. The fibrin clot was well filled with gas bubbles and also floated; however, no peptonization of the coagulum, even after a month, was noted. The hemoglobin was changed to a brownish, granular mass. The cultures had a strong butyric acid odor. The bacteria lost their virulence rapidly in this medium.

In Milk.—The fresh milk was kept in test-tubes, Erlenmeyer flasks, or fermentation tubes. Anaerobiosis was obtained by stratification with sterile oil, or by absorption of the oxygen, or by vacuum, etc. In all cases the changes were the same. In from 4 to 15 days the milk coagulated smoothly. A small amount of turbid whey was pressed out from the clot. After about 15 days the whey was clear and more abundant. The casein clots became attached to the walls of the tubes or flasks, and were shrunken. The curd was smooth and cheesy in a few instances only. A few holes, due to gas bubbles, were present in the tubes which had been inoculated with the infected heart-blood. The effect of the blood was probably like that of the addition of tissues. When sterile tissues had been added to the medium, quite different results were noted. The coagulation took place in from 24 to 48 hours and was associated with abundant gas production. The whey was more excessive, and had a deep yellowish tinge. The casein clots floated on it and frequently the entire coagulated mass lay directly under the cream, the whey being about two-thirds of the contents of the tubes or flasks. The clots were all bloated by gas bubbles. In from 10 to 20 days, shrinkage of the coagula also took place. The results were constant and apparently not influenced by the age of the cultures from which transplants were made or the source of material, whether animal or culture. The amount of inoculated material or culture did not alter the result as long as fresh milk, directly collected from the cow, was used. Old milk coagulated slowly, and less vigorous gas production was the rule. In 4 days about 4%, and in 30 days about 6.5%, acid was produced. The odor was always rancid, typically like that of butyric acid, never disagreeable or foul. In the tubes without tissues the odor was slight, as compared with those with tissues. The cultures were carefully studied for peptonization of the curd.

Even in cultures one and one-half years old, in which desiccation had been prevented, no liquefaction of the casein clots or flakes occurred.

In Brain Medium According to v. Hibler.—In 24 hours the semi-liquid mass was usually split by gas bubbles, which, when shaken, rose to the surface and caused frothing of the upper layers of the medium. The odor was markedly rancid, like that of butyric acid. Microscopically, there were present rods with and without spores, strongly gram-positive, containing granulose. In about 5 days the color of the brain emulsion in the deeper layers was bright-pinkish, and only a zone of about 0.8 to 1.0 cm. was grayish-brown. Over 50 tubes, inoculated with material from subcultures or directly with animal material, were kept for 1 year; no changes were noted, with the exception of a broadening of the upper grayish zone of oxidized brain material and, in some tubes, an intensification of the pink color to deep red.

TABLE 1
TESTS FOR THE FERMENTATION OF CARBOHYDRATES

Organism	Dextrose*	Lactose*	Saccharose†	Maltose*	Sorbite‡
Bacillus isolated from hogs	40% 1.8% —3.2% Heavy sedi- ment	20% 2.6% —3.9% Heavy sedi- ment	Bubble (0.9% —1.2%) Slight sedi- ment	35% 2.2% —2.7% Heavy sedi- ment	Bubble (1.0%) Heavy sedi- ment
"Vibron septicque" de Pas- teur	45% 1.8% Heavy sedi- ment	40% 2.5% Heavy sedi- ment	Bubble (1.5%) Slight sedi- ment	40% 2.1% Heavy sedi- ment	Bubble (0.7%) Slight sedi- ment
Blackleg (Strain 2, Munich)	25% 3.5% Heavy sedi- ment	35% 2.3% Heavy sedi- ment	Bubble (1.3%) Slight sedi- ment	30% 2.6% Heavy sedi- ment	Bubble (1.3%) Slight sedi- ment
Control	0.2 %	0.2 %	0.2%	0.15%	0.9%

* Kahlbaum. † Merck. ‡ Bausch and Lomb.
The figures give the gas and acid reactions.

The reaction was decidedly acid and remained so for the entire period of observation. Some titration tests⁴ with 1:100 normal sodium hydroxid gave the following results:

First day	0.9% acid
Third day.....	1.9 "
Fifth day.....	2.4 "
Eighth day.....	5.4 "
Eighteenth day.....	6.6 "

In this medium the organism remained alive and virulent for a period of over 2 years. Subcultures could always be successfully obtained and in some instances this medium was also used to procure pure subcultures by heating 48-hour-old brain cultures (in which spores were numerous) for 30 minutes, at 80 C.

In Coagulated Blood Serum, Yolk, and Egg Albumin, and Ascitic Fluid, with and without 1% Glucose.—The organisms grew abundantly, with little or extensive gas formation, depending on the presence of fermentable carbohydrates. The coagulated protein mass, under these conditions, was split into

smaller clumps and a yellowish fluid expressed. The reaction of this fluid was always strongly acid. Peptonization of the clots has not been recorded even after a period of observation of 6 months. Nor were there present any changes in color, particularly in the yolk media. The odor resembled that of butyric acid.

*In Potato, Plain, and Immersed in Sodium Carbonate Solution (0.5-2.5%.—*On the ordinary potato medium no satisfactory growth could be obtained. In media containing less than 1% sodium carbonate a moderate growth was noticed in the slight gas production and the change of the medium to an acid reaction. In higher concentrations of the alkalis no growth was ever seen; apparently the spores did not germinate.

Biochemical Activities.—Indol was not formed by the organism in peptone solutions when tested by the Ehrlich -paradimethylamidobenzaldehyde method.

TABLE 1.—*Continued*
TESTS FOR THE FERMENTATION OF CARBOHYDRATES

Arabinose*	Xylose*	Rhamnose‡	Mannite†	Dulcite*	Glycerin†	Plain
None (0.8%)	Bubble (0.8%)	Bubble (0.8%)	Bubble (1.0%)	Bubble (1.0%)	Bubble (1.0%)	Bubble (1.0%)
Slight sediment	Slight sediment	Slight sediment	Slight sediment	Slight sediment	Slight sediment	Slight sediment
Bubble (1.0%)	Bubble (1.4%)	Bubble (0.5%)	Bubble (1.6%)	Bubble (1.1%)	Bubble (0.8%)	Bubble (0.8%)
Slight sediment	Slight sediment	Heavy sediment	Slight sediment	Poor sediment	Slight sediment	Slight sediment
Bubble (1.0%)	Bubble (1.5%)	Bubble (1.0%)	Bubble (1.5%)	Bubble (1.2%)	Bubble (1.0%)	Bubble (0.9%)
Slight sediment	Sediment	Sediment	Slight sediment	Very slight sediment	Sediment	Slight sediment
0.2%	0.2%	0.2%	0.8%	0.8%	0.8%	0.8%

Sulphurated hydrogen was demonstrated by suspending a piece of filter paper, moistened with lead acetate solution, in the space between the cotton plug and the broth culture, which was kept in a Buchner's tube. The paper was blackened in from 48 to 72 hours. Cultures on solid agar media containing lead acetate or ferrosulphate (1:1000) grew freely, with extensive gas formation, but were never turned black; the reaction of the medium was always acid and, therefore, iron sulphid was not precipitated.

Neutral red, litmus, methylene blue, sodium indigosulphate, when added to solid or liquid media, are reduced.

Fermentation of Carbohydrates.—For the fermentation tests the method of Bahr¹⁰ was used. Large Durham tubes each containing 1% of one of the various carbohydrates in Liebig's broth were inoculated, by means of pipets, with the sediment of the 48-hour-old cultures of the organisms to be tested. The test-tubes were kept in a Novy jar under vacuum-hydrogen and pyrogallie acid for 5 days at 37 C. Aerobic control-tests were prepared simultaneously. The tests were repeated several times. The results are tabulated in Table 1. The organism under discussion fulfills all the reactions characteristic for Group 1 in Bahr's¹⁰ classification. It cannot be separated from the blackleg

organisms or the "vibrio septique" of Pasteur by means of the fermentation tests. The growth was extensive in the tubes where fermentation occurred; the gas and acid production fluctuated; noteworthy was the fact that small amounts of gas and acid were also produced in plain broth, probably as a result of catabolic acid on the protein substances. In 4% glucose broth, in Smith's fermentation tubes, 60-90% gas is produced. The analysis gave a gas formula of $2\text{H}/\text{CO}_2$ to $4\text{H}/\text{CO}_2$. In numerous tests these proportions were constantly found. The production of ethylalcohol, tested according to Foth,²⁸ was found in 2 tests to be constant.

Spore Resistance.—For the determination of spore-resistance to heat, the method of v. Hibler²⁰ was used. The temperature of the steam at Berkeley was between 99 and 99.5 C. Brain media in thin, long test-tubes were used for subcultures. The resistance to the heat of boiling water was very low, varying between 5 and 8 minutes. An exposure of 10 minutes to a temperature of 100 C. invariably killed the organism. The results of the various tests are tabulated in Table 2. At a temperature of 75 C., the spores remained alive

TABLE 2
RESISTANCE OF SPORES TO HEAT

Spores (Inoculated into Brain Media)	Growth (+) During Exposure to 99 - 99.5 C. From 2 to 20 Minutes								
	2 Min.	4 Min.	5 Min.	6 Min.	7 Min.	8 Min.	10 Min.	15 Min.	20 Min.
Two-day-old blood broth culture.....	+	+	+	+	—	—	—	—	—
Two-day-old beef broth culture.....	+	+	—	—	—	—	—	—	—
Twenty-four-day-old brain medium culture	+	+	+	+	—	—	—	—	—
Eight-day-old milk culture.....	+	+	+	—	—	—	—	—	—
Three-day-old brain medium culture..	+	+	+	—	—	—	—	—	—
Four-day-old brain medium culture...	+	+	+	—	—	—	—	—	—
Six-day-old brain medium culture.....	+	+	+	+	+	—	—	—	—

even after an exposure of 5 hours (longest period tested). In organ material (muscle), when kept cool, the spores remain unaltered for years (2½ years longest period tested). In broth, blood, milk, and agar cultures kept at 37 C. for longer than 4 weeks, a reduction in the number of living spores was frequently noted; in some instances, the organism even died out. Brain media (in numerous observations) were apparently more protective for the spores.

PATHOGENICITY

The pathogenicity of the bacillus was tested with animal material (muscle of the hogs) and also with pure cultures. The material inoculated was always previously sterilized from all non-spore-bearing organisms, as already indicated under the heading of cultural characteristics. The cultures varied for the different tests and are mentioned separately in Table 3. The dead animals were either examined immediately after death, or the cadavers were well kept, on ice. Each animal was carefully examined bacterologically; aerobic and anaerobic cultures were prepared.

Primary Animal Inoculations.—Aside from the inoculation of guinea-pigs for the preparation of pure cultures, rabbits, pigeons, mice, rats, and hogs were

infected with spore material from the muscles of Hogs 1 and 2. Inasmuch as the results are the same in the small animals as those obtained with pure cultures, the inoculations and the autopsies on these animals, with the exception of the hogs, are summarized in Table 3. The muscles from Hog 16037 were dried, as explained, and used for the primary inoculation experiments on the small laboratory animals, mentioned in Table 3.

These experiments were undertaken primarily with the idea of establishing a diagnosis, since, according to current opinions, all laboratory animals are very susceptible to malignant edema as compared with blackleg. The amounts chosen for the inoculations were as small as possible so that a marked contrast might be observed, particularly in rabbits. According to Table 3, the organism is pathogenic for guinea-pigs, rabbits, mice, pigeons, and rats. With the fresh material, uniformly fatal results were obtained. One year and a half later, however, the pathogenicity of the organism for rabbits and rats had noticeably diminished and it behaved in many respects like *Bacillus chauvæi*. The inoculation of pure cultures is therefore of more importance and the entire question is consequently best discussed in connection with the experiments along these lines.

Inasmuch as the anaerobes commonly lose their virulence quickly, it was considered advisable to conduct experiments reproducing the disease as observed in the hogs from which the material was collected, before the organism had been studied in detail and the absolute purity of the cultures ascertained. Two experiments, of which one was successful, are reported in detail. With a comparatively large dose of dried muscle a large sow was readily infected by subcutaneous application of the material behind the ear. The course of the infection resembled closely that reported from the spontaneous cases, and the post-mortem and bacterioscopic findings correspond exactly with those in Case 2. The bacillus, therefore, is pathogenic for hogs and is undoubtedly capable of producing the lesions resembling blackleg in cattle. Inoculation experiments with pure cultures were carried out about 2 years later, at a time when the strain had already lost its virulence. These tests were not as successful, as will be shown, and therefore the experiment on Hog 2 is of importance for the later discussion of the entire problem of infection with this anaerobic organism.

WHITE BARROW 16045.—Weight, 200 pounds. Inoculated on Sept. 10, 1912, with 1 c.c. of a suspension of dried muscle from Hog 1 (0.1 gm. to 10 c.c. of salt solution) subcutaneously back of the left ear. On September 11, the animal showed a hot, edematous, but not emphysematous, swelling. It appeared depressed and did not care for food. No temperature reaction was recorded. On September 14 the swelling had disappeared. The hog was killed several weeks afterwards and found to be normal.

BLACK AND WHITE Sow 16037.—Weight, 230 pounds. Inoculated, Sept. 14, 1912, with 10 c.c. of a suspension of muscle from Hog 1 (diluted as for Hog 16045) subcutaneously on the back of the left ear. The temperature curve is shown in Chart 1. On September 16, a swelling of the size of two fists, had developed behind the left ear. The animal was noticeably sick. On September 17, the swelling had spread along the submaxillary and cervical region; the hog was unable to swallow and showed also difficulties in breathing; the mucous membranes of the head were slightly cyanosed. On September 8, the hog died during the morning, about 88 hours after inoculation.

The autopsy was made 3 hours after death. The skin of the abdomen was slightly purplish. In the left auricular region, extending to the shoulder

TABLE 3
INOCULATIONS WITH HEATED, DRIED MUSCLE OF HOG 1

Animal	Material Inoculated	Seat of Inoculation	Died After	Time Elapsing Between Death and Autopsy
Guinea-pig ...	0.3 c.c. of muscle = 0.03 gm. powder	Subcutaneous	18 hr.	1½ hr.
Guinea-pig ...	0.5 c.c. of muscle = 0.03 gm. powder	Subcutaneous	14¾ hr.	10 hr.
Rabbit	0.5 c.c. of muscle = 0.5 gm. powder	Subcutaneous
Mice (2)	0.1 c.c. of muscle = 0.001 gm. powder	Subcutaneous	38 and 40 hr.	2½ hr.

INOCULATIONS WITH HEATED, DRIED MUSCLE MATERIAL OF HOG 16037

Guinea-pig ...	0.5 c.c. of muscle = 0.5 gm. powder	Subcutaneous	16 hr.	2 hr.
Rabbits (6)...	0.5-1.0 c.c. of muscle 1-0.5 gm. powder	Subcutaneous	14-20 hr.	½-3 hr.
Pigeons (2)...	0.5 c.c. of muscle = 0.5 gm. powder	Subcutaneous	14 hr.	Immediately examined

INOCULATIONS WITH HEATED, DRIED MUSCLE OF HOG 2

Guinea-pig ...	0.2 c.c. of muscle = 0.02 gm. powder	Subcutaneous	About 16 hr.	Immediately examined
Pigeon	0.2 c.c. of muscle = 0.02 gm. powder	Intravenous	20 hr.	1 hr.
Rat	0.5 c.c. of muscle = 0.5 gm. powder	86 hr.	5 hr.
Rat	0.1 c.c. of muscle = 0.1 gm. powder	Alive
Rat	0.5 c.c. of muscle; 1:2 powder	Subcutaneous	Alive; discarded 6/12/14
Rat	0.5 c.c. of muscle; 1:2 powder	Subcutaneous	Alive; discarded 6/12/14
Rabbit	0.5 c.c. of muscle; 1:2 powder	Subcutaneous	Discarded 6/12/14
Rabbit	1.5 c.c. of muscle; 1:2 powder and 4 drops of lactic acid	Subcutaneous	Discarded 6/12/14
Rat	0.1 c.c. uterus contents of Rabbit T. 2	Subcutaneous	60 hr.	12 hr.
Rat	0.1 c.c. uterus contents of Rabbit T. 2	54 hr.	20 hr.

TABLE 3.—*Continued*
INOCULATIONS WITH HEATED, DRIED MUSCLE OF HOG 1

Lesions	Microscopic Findings	Cultural Results
Serous, hemorrhagic edema with gas bubbles; necrosis at seat of inoculation	Short rods with spores; on peritoneum, filaments	Pure culture from heart-blood and edema
Serous, hemorrhagic edema with gas bubbles; necrosis at seat of inoculation	Short rods with spores; on peritoneum, filaments	Pure culture from heart-blood and edema
Developed a large local infiltration, but recovered completely
Serous, hemorrhagic edema	Short rods and numerous clostridium forms; long filaments on liver	Pure cultures from heart-blood and edema

INOCULATIONS WITH HEATED, DRIED MUSCLE MATERIAL OF HOG 16037

Serous edema in inguinal region	Short rods only; on peritoneum, filaments	Heart sterile; edema positive anaerobically
Hemorrhagic edema; fluid in peritoneum; muscles of abdomen very soft and friable; very few gas bubbles	Short, spore-bearing rods; on peritoneum, filaments	Edema and peritoneum positive; heart-blood positive in one animal
Necrosis and gas-phlegmon at seat of inoculation; hemorrhagic myositis	Distorted, spore-bearing rods; on liver surface, filaments	Muscle, anaerobically, positive; heart-blood sterile

INOCULATIONS WITH HEATED, DRIED MUSCLE OF HOG 2

Serous, hemorrhagic edema with muscular emphysema	Short rods only; filaments rare	Edema pure culture; heart sterile
Necrosis hemorrhages, and edema of pectoral muscle; peritoneal fluid	Short rods and clostridium forms	Muscle positive; heart sterile
Ulcer at seat of inoculation; serous, slightly hemorrhagic edema; no gas bubbles	Well-spored rods; on liver surface, filaments	Gluteus muscle positive
Slight edema at seat of inoculation		
Slight local edema at seat of inoculation		
Slight local edema at seat of inoculation		
Necrosis at seat of inoculation....		
Slight local edema.....		
Necrosis at seat of inoculation; slight serous edema; general congestion; duodenitis; lung edema	Spore-bearing rods in muscles and edema; on surface of liver, filaments in coils	Heart-blood and edema, anaerobically, positive
Marked serous, hemorrhagic edema; no necrosis; same as Rat 16	Spore-bearing rods in muscles and edema; on surface of liver, filaments in coils	Ditto; aerobically, heart-blood gave <i>B. coli</i>

blade, was a diffuse edematous swelling, which on section proved to be due to a yellowish serogelatinous infiltration of the subcutaneous and intermuscular tissues of the places mentioned. At several places a few gas bubbles were seen. In the tracheocephalic, deltoid, and trapezius muscles, several areas of the size of a small hand were dark-brown in color, and moist, and some places were emphysematous, floating in water. On incision these areas crackled, and the muscle fibers were separated by gas bubbles; the center of these muscles was patchy, yellowish and necrotic. The lymph vessels in the perimysium were very marked. Only the muscles of the left side were affected. The submaxillary and prescapular lymph-nodes were as large as plums, soft, juicy, and hyperemic on section. The trachea, larynx, and pharynx showed a slightly thickened, edematous mucous membrane which was covered with a large amount of mucus and deeply injected. In the mediastinum was a gelatinous infiltration; a few atelectatic foci in the anterior lung lobes; a few hemorrhages on the pleura.

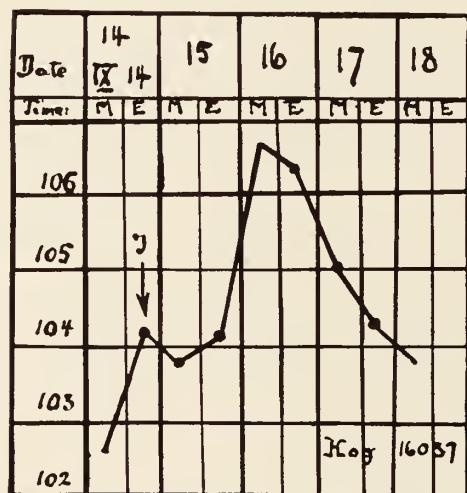


Chart 1.—Temperature curve for Hog 16045.

The fundus of the stomach was hyperemic; spleen, soft and small; liver, normal; other organs normal. The pericardiac sac contained about 15 c.c. of fluid, which was slightly turbid. The blood was well clotted; no abnormalities observed on the endocardium.

The bacteriologic examination resulted as follows—

Edema of the subcutis: Mostly long, very few short, rods in aggregations of pairs, some in filaments, distinctly gram-positive. Some were granular with spore formation. Spores were rare, also the lemon- or whetstone-shaped clostridium forms. Shadows of rods were numerous and granulose in a few clostridium forms.

Muscle juice: Clostridium forms less frequent than in spontaneous cases; endospores numerous in rods.

Pericardiac fluid: A few short rods, single, and in pairs.

Liver surface and peritoneum: No organisms noted.

Liver parenchyma: A few short rods, also a few filaments.

Cultures: (1) Heart-blood, aerobically, was sterile (enriched in broth); anaerobically, the bacillus was isolated in pure culture. (2) Edema and muscle, aerobically, gave cocci; anaerobically, the bacillus and cocci. (3) Pericardiac fluid, anaerobically, the bacillus; aerobically, sterile. Liver, aerobically, gave intestinal organisms; anaerobically, the bacillus, mixed with intestinal organisms and cocci.

Animal Inoculations with Pure Cultures.—The primary animal inoculations did not permit the injection of an exact dosage of the bacteria or spores. For a comparative study with similar organisms which have been described, only the results obtained with pure cultures are worth consideration. Most of the results are tabulated, as far as they are of interest and deal with pure infections. Some detailed reports are added for each animal, to convey to the reader an exact picture of the methods, findings, and the amount of work which were connected with these tests. The results have not been uniform, probably, for the most part, because of the gradual decrease of virulence of the strains used. Various types of cultures of varying ages were employed,—factors which also had an influence on the results and their interpretation. There is not however a different susceptibility in different species of animals, as is a well established fact for *Bacillus chauvæi*. Individual fluctuations, on the other hand, seem to account for some results. It will be shown that a certain amount of care has to be observed in using the results for differential diagnostic purposes.

Tests on Guinea-Pigs.—Subcutaneous inoculations only were used, the amounts of the various cultures varying from 0.1 to 0.5 c.c. In every instance, the infection was fatal, the animals dying in from 18 to 68 hours after injection. The dosage apparently had no influence on the duration of the disease; the age of the culture and the medium used were however important factors; the addition of sodium formate or the cultivation in milk apparently reduced the virulence.

The inoculated guinea-pigs became less active after varying periods of time following the injections. After 12 to 16 hours they sat uniformly quiet in the cages, their fur ruffled; at the seat of the injection, a well-defined, edematous swelling developed, which was hot, and showed a bluish discoloration in some instances. In a few cases only, crepitation was noticed. The swellings spread in the following hours and the animals grew very weak, finally lying on one side, for varying periods, before death.

The autopsy revealed the usual well-known lesions of a gas-phlegmon at the seat of inoculation. The skin was strongly infiltrated; on incision, a small amount of reddish fluid oozed out. The subcutaneous and intermuscular connective tissues along the abdomen, thorax, and inguinal and subscapular spaces were infiltrated with a serous, slightly hemorrhagic fluid; the appearance of the tissue was jelly-like and contained numerous, small gas bubbles. Between the fascia, gas bubbles were also frequently noticed. The muscles and the fascia bore, occasionally, small and large hemorrhages. At the seat of inoculation, the subcutaneous tissue was yellowish, gray, soft, friable, and necrotic.

The muscles of the abdomen were very soft and "soaked" with fluid, which frequently retained small gas bubbles. The inguinal and subscapular lymph-nodes were slightly enlarged and very soft and edematous. In the abdominal and thoracic cavities were varying amounts of fluid; in the abdomen, it was turbid and grayish in color. The serous membranes were frequently covered with small hemorrhages; the parietal peritoneum was deep-reddish, shiny, and in some places grayish. In some instances, areas with streaks or gas bubbles

were very prominent. The small intestines showed injection of the serosa, contained a bile-stained liquid, and presented catarrhal inflammation of the mucous membrane. The other organs were not constantly changed. The spleen was small. The liver was frequently dark-brownish; in a few cases, when the cadavers were not examined immediately, the organ was mottled by yellowish areas of a spongy, foamy, infarct-like character. The kidneys were deeply reddened and soft. In pregnant animals, the involvement of the uterus and fetus was very marked. The hemorrhagic-necrotic character of the uterine membrane following abortion was the most marked anatomic lesion of the abdominal cavity.

TABLE 4
EXPERIMENTS ON GUINEA-PIGS

Weight of Guinea-pigs in Grams	Material Inoculated	Seat of Inoculation	Died After	Time Elapsing Between Death and Autopsy
450	Blood broth, 48 hr. old.....	0.1 c.c. subcutaneously	18 hr.	6 hr.
375	Blood broth, 48 hr. old.....	0.1 c.c. subcutaneously	22 hr.	4. hr.
393	Blood broth, sodium formate culture, 1 day old	0.1 c.c. subcutaneously	68 hr.	4. hr.
690	Sodium formate broth, 3 days old, heated	0.2 c.c. subcutaneously	42 hr.	3½ hr.
319	Sodium formate broth, 9 days old, heated for 2 hr. at 76 C.	0.3 c.c. subcutaneously	36 hr.	8 hr.
659	Sodium formate broth, 3 days old, heated for 3 hr. at 76 C.	0.2 c.c. subcutaneously	40 hr.	6 hr.
480	Milk culture, 28 days old.....	0.5 c.c. subcutaneously	36 hr.	9 hr. at room temperature

The thoracic organs were usually not affected. The lungs were pale and perhaps a slight edema of the lung was present. The heart-blood was poorly clotted and dark.

Microscopic preparations, made from the edema, demonstrated the organisms in their various forms. The internal organs usually contained the bacteria in very small numbers, either in pairs or in aggregations of filaments. The liver and peritoneal surfaces revealed the bacteria always in aggregations of filaments and threads.

The inoculation experiment on Guinea-Pig T.-40 is reported in detail, to illustrate the foregoing statements.

GUINEA-PIG T.-40.—Weight, 659 gm. Inoculated subcutaneously, May 16, 1914, with 0.2 c.c. of 3-day-old sodium formate (0.5%) glucose (1%) broth culture heated for 3 hours at 76 C. (strain from Guinea-Pig T.-16). After 20

hours the animal was apparently much distressed; its fur was ruffled; along the abdomen and towards the thorax there was a hot, edematous, well-defined swelling. The guinea-pig died in 40 hours after inoculation.

The autopsy was made 6 hours post mortem (4 hours on ice). On shaving the hair on the abdomen from the blue-reddish hide, a reddish fluid oozed out. In the inguinal folds marked crepitation was felt. On incision a broth-like serum escaped from the fascia and intermuscular tissues. In the inguinal and subscapular spaces along the neck the edema was tinged deep-reddish. All the muscles of the abdominal wall were rich in fluid; the intermuscular connective tissue on the hind leg showed numerous petechiae. The

TABLE 4.—*Continued*
EXPERIMENTS ON GUINEA-PIGS

Lesions	Microscopic Findings	Culture Tests
Serous edema; hemorrhagic myositis of pectoral and femoral muscles; catarrhal enteritis	Short rods; clostridium forms in edema and muscles; aggregations of filaments on liver surface—typical findings	Heart-blood positive; anaerobically, sterile
Serous, hemorrhagic edema; intermuscular hemorrhage; catarrhal enteritis	Short rods; clostridium forms in edema and muscles; aggregations of filaments on liver surface—typical findings	Heart-blood positive; anaerobically, sterile
Pronounced hemorrhagic, serous, gaseous edema; hemorrhagic myositis, and necrosis at seat of inoculation	Short rods; clostridium forms; terminal spores; long rods on liver surface	Heart-blood, anaerobically, positive
Hemorrhagic, serous edema; hemorrhagic, ichorous metritis; liver infarct	Mostly long rods and a few clostridium forms and spores in uterus and liver	Uterus, anaerobically, positive
Hemorrhagic, serous, gaseous edema; foamy liver; lung edema	Short and pleomorphic rods in edema; numerous spores	Heart-blood and liver, anaerobically, positive
Typical (see separate report).....	Typical	All organs positive
Hemorrhagic, serous, gaseous edema; enteritis; lung edema; foamy liver and spleen	Rods of different sizes with and without spores; filaments on liver	Heart-blood and edema, anaerobically, positive

fascia and the finer tissue membranes were lifted up by gas bubbles. At the seat of inoculation the subcutaneous tissue and the superficial muscle layers were yellow-grayish and friable.

In the peritoneal cavity was a small amount of turbid, reddish fluid. The abdominal walls were moist; the parietal peritoneum was bright red and retained gas bubbles. The small intestines were distended by gas; the serosa was injected; the contents in the duodenum were liquid, and yellowish in color. The stomach was empty; in the fundus, numerous hemorrhages and superficial erosions, and beginning auto-digestion. The liver was light-brownish; some areas were anemic and compressed by gas bubbles; on section the color was mottled. The spleen was small, but dark. The kidneys were brownish and showed localized injection. Bilateral pregnancy existed; the uterine fluid was reddish; the placenta, hemorrhagic.

In the thoracic cavity, a few drops of fluid were present; the lungs were pale but edematous; a few petechiae on the tracheal mucous membrane; the heart was light-brownish, and the blood was well coagulated in both ventricles.

The bacteriologic examination resulted as follows—

Edema of subcutis and uterus: Gram-positive rods; cigar-shaped and clostridium forms rare; a few spore-bearing rods; long rods very thin, gram-negative. Granulose formation typical in a few clostridium forms.

Muscle juice: Short, gram-positive rods, a very few spore-bearing.

Peritoneal lining: Long filaments of rods; no spores.

Liver: Long rods and aggregations in pairs.

Kidney, spleen, and heart-blood: A few gram-positive rods, some in aggregations of filaments.

Cultures: Anaerobically, the edema, the organs, and the heart-blood were positive; aerobically, they were sterile.

TABLE 5
EXPERIMENTS ON RABBITS

Weight of Rabbit	Material Inoculated	Seat of Inoculation	Died After	Time Elapsing Between Death and Autopsy
2350	Blood broth culture, 48 hr. old....	1 c.c. subcutaneously on back
2200	Blood broth culture, 48 hr. old....	0.5 c.c. intravenously	10 min.	Immediately examined
2670	Blood broth culture, 12 days old..	0.5 c.c. intravenously	About 88 hr.	7 hr.
1950	Blood broth culture, 4 days old...	1.5 c.c.
2300	Blood broth culture, 0.5, 1.0, 2.0, respectively	Intravenously	14 hr.	8 hr.

In Table 4, the experiments on guinea-pigs with pure cultures are summarized.

Tests on Rabbits.—Experiments on rabbits with pure cultures are of the greatest interest inasmuch as they frequently permit a diagnosis in one or the other direction. Most of the workers with anaerobes have an idea that the rabbit is relatively resistant to an infection with *Bacillus chauvæi*. On the other hand, it is very susceptible to organisms belonging to the malignant edema group of bacteria.

The tests which the writer has been able to carry out apparently did not entirely support this view; the first inoculations showed a marked susceptibility on the part of the rabbit to the organism isolated from the hogs. Subcutaneous inoculation with cultures failed to verify the first results. Only one explanation can be offered for this discrepancy, namely, the loss in virulence. Most of the cultures used for these tests were prepared with muscle material already 1½ years old, and it is a known fact that such anaerobes lose their virulence gradually, so that the difference in pathogenicity for certain species

of animals becomes more and more pronounced. On the other hand, some of the results which are tabulated in Table 5 bear out the findings of Ghon and Sachs, who first described the organism, found in this instance in blackleg of hogs.

Subcutaneous inoculations in small doses caused no reaction whatever; in large doses, a well-defined, edematous swelling developed, which resulted in necrosis of the skin and ulcer formations with much retarded healing. The injected organisms could be isolated from the necrotic tissue.

Intravenous inoculations were fatal in two instances. One experiment is not conclusive, however, as the rabbit had already received several other injections and probably succumbed to an anaphylactic shock caused by the foreign protein introduced with the blood cultures.

The only successful experiment is reported herewith, in detail. It shows that the septicemic character of the infection caused a picture very different

TABLE 5.—*Continued*
EXPERIMENTS ON RABBITS

Lesions	Microscopic Findings	Culture Tests
Healed ulcer on the back. Discarded 6/15/14 Negative; probably embolic.....		
Sero-fibrinous pleurisy and pericarditis; hemorrhagic-ichorous metritis; spleen tumor; kidney infarcts; myositis; local edema. Discarded 6/15/14	Long and short rods with spore formation; typical	Heart-blood, uterus, etc., anaerobically, positive
Lung emphysema; hemorrhagic metritis; anaphylaxis; pregnancy	Short rods and clostridium forms in uterus; long rods in heart-blood	Heart-blood and uterus, anaerobically, positive

from that resulting from subcutaneous injection; namely, sero-fibrinous exudates on all serous membranes, and localization of the gas-phlegmon in the muscles and the uterus. The picture is strikingly similar to the one seen occasionally in infections with *Bacillus chauvæi* in cattle. The microscopic examination revealed a picture different from the usual, in the most remarkable richness in shape and varieties of sporulated and non-sporulated, free granulose-bearing forms in the uterus of the rabbit.

RABBIT J.-2.—Weight 2,400 grams. Inoculated intravenously with 0.5 c.c. blood broth culture (12-day-old strain from Hog 2). Died about 88 hours after inoculation. The autopsy was made 7 hours post mortem. The animal was bloated, with slight rigor mortis. Subcutaneous tissue, moist; blood vessels well injected; mammary glands diffusely dark-red, showing numerous small and large interglandular hemorrhages.

In the abdominal cavity, a considerable amount of reddish fluid was present. The serous lining of cecum and colon was covered with petechiae; in the peritoneal lining in the inguinal region, diffuse hemorrhages, suffusions, and petechiae.

The uterus and both horns were brownish-red and distended by gas. On opening there were in the left horn two placentas with black-brownish fetal envelopes, surrounded by a frothy, purulent exudate; in the right horn, one placenta, black in color. The uterine membrane was diffusely reddened, covered by pus and fibrin clumps; in some areas, it was lifted by a few large gas bubbles. The external os was smeared with clumps of purulent-necrotic material. Vaginal membrane showed pus and fibrin flakes and necrotic tissue; longitudinal streaks of hemorrhages very prominent. The serosa of the urinary bladder was reddened, showing some petechiae.

The spleen was enlarged; edges rounded; pulp, black and soft. Liver was slightly enlarged, pale in color and soft; bile, liquid and light-greenish. Right kidney was light-brownish with a yellowish area, the size of a pea, surrounded by a bright-red zone of demarkation; medulla, purplish in color. Left kidney, the same, with two well-demarcated, anemic infarcts. Serosa of the duodenum, covered with petechiae; contents, liquid and greenish. Colon contained semi-solid contents; mucous membrane slightly swollen.

The right pleural cavity contained about 20 c.c. of a serous-hemorrhagic fluid. Costal pleura, covered with petechiae along ribs. Anterior right and left lobe loosely attached to pericardiac sac by fresh fibrin threads. Pericardium contained a few drops of fluid; it was covered by fibrin in streaks. Both ventricles, at maximal distention; blood, well-coagulated; in left ventricle, a few hemorrhages beneath the valves. Myocardium, soft and pale. All lobes of the lungs mottled; foam present in trachea and bronchi; mucous membrane injected, showing multiple petechiae and suffusions. Thymus dark-grayish, covered with petechiae. Gastrocnemic muscle, spongy, dark in color, and dry. Popliteal lymph-nodes, enlarged, moist, and hemorrhagic on section.

The bacteriologic examination resulted as follows—

Hanging drop of uterus exudate: Very long rods; oval-shaped forms with and without spores. Short forms very actively motile, some showing slight "granulose" formation; most rods gram-positive; spores confined to clostridium forms.

Pericardiac and pleural exudates: Long and short gram-positive rods; a few spore-bearing, clostridium forms.

Popliteal lymph-nodes: Short, gram-positive rods in pairs; no spore-bearing, spindle-shaped forms.

Liver: Short and long rods on the surface; in parenchyma, mostly spore-bearing, clostridium forms.

Heart-blood: Short, gram-positive rods; no spore-bearing rods.

Cultures: (1) Anaerobically, the spleen, uterus, pericardiac exudate, pleural fibrin, and heart-blood yielded heavy growth; (2) aerobically, heart-blood, uterus, pleural exudate, and spleen were sterile.

Tests on White Rats.—The transmission experiment with muscle material revealed the fact that white rats are very resistant to infection with the bacillus isolated from hogs. With pure cultures the results were conclusive. Small doses applied subcutaneously or intramuscularly caused the local reactions already described for the rabbits, but in no instance did death result from the infection. An intramuscular injection of a large dose proved fatal; the young rat succumbed to the infection in 16 hours. The subcutaneous edema was slight; the hemorrhagic, spongy character of the muscle resembled greatly the lesions seen in the hog; a catarrhal inflammation of the small intestines was also present. The microscopic findings were as usual.

Tests on White Mice.—In 2 experiments the bacillus proved to be pathogenic for white mice. Very small doses, by subcutaneous application, caused

the death of the animals in from 18 to 20 hours. The anatomic lesions consisted in a slight serous infiltration of the subcutis, marked enteritis, and slight degenerative changes of the parenchymatous organs. The bacteria were rare in the internal organs. The writer has the impression that the death and the lesions in the animals were mostly due to the toxins which were inoculated with the broth cultures. Further experiments, to prove this statement, will be reported farther on in this paper.

Tests on a Pigeon and a Chicken.—To confirm the results obtained with muscle material, one pigeon was inoculated intramuscularly; the injection of 1 c.c. caused death in about 18 hours. The changes, in the form of a marked gaseous myositis, are described in detail in the appended report.

PIGEON A.—Inoculated, intramuscularly in right pectoral muscle with 1 c.c. of a 48-hour-old blood broth culture. Died in about 28 hours. Examined 2 hours post mortem: The right pectoral region was puffed and crepitant, discolored bluish. On incision, the subcutis and muscle were found infiltrated by a serous fluid mixed with gas bubbles. The muscles were separated by a gelatinous, gaseous mass on the fascia. The muscle, on section along the needle tract, throughout a circular area of about 2 c.c. was hemorrhagic, very friable and moist; several bundles were yellowish and necrotic, diffusely dark. The entire muscle tissue was spongy and separated by gas bubbles. The duodenal loop was deeply injected, the contents blood-tinged, liquid; the mucous membrane swollen; spleen small; liver dark-brownish and mottled in areas. A slight edema of the lung was present.

The bacteriologic examination resulted as follows—

Muscle: Numerous gram-positive rods; clostridium and cigar-shaped forms, common; sporulation, very extensive; also a few free spores. "Granulose" present in oval forms.

Liver and intestinal surface: Short and long rods, in pairs, and aggregations of filaments; no sporulated forms.

Heart-blood: Numerous gram-positive, short, plump rods; no spores.

Cultures: Heart-blood, muscles, liver, and kidneys, anaerobically, positive; aerobically, heart-blood and muscle, sterile. Kidneys show cocci.

A chicken inoculated with the same amount did not develop the slightest local reaction, nor did it succumb to an infection.

Tests on Hogs.—The writer has already discussed the successful reproduction of the anatomic picture of the lesions found in the hogs that had apparently succumbed to a blackleg-like infection, by subcutaneous injections of original muscle material. The experiment did not permit of any conclusion, however, nor did it furnish evidence for the conception advanced that the bacillus causing the retropharyngeal gas-phlegmon had entered by the tonsils or the upper digestive tract. The tests with pure cultures had therefore only the object of reproducing, if possible, the clinical picture and the anatomic lesions by direct intratonsillary injections. Unfortunately, these tests could only be carried out with material which had already been kept for over a year and which had lost its virulence, as was shown elsewhere. The results are not absolutely conclusive.

Hog 1256.—Weight, 8,880 grams. Inoculated, June 19, 1914, with 1.5 c.c. blood broth culture (40 hours old) in the left tonsil. The tonsil was slightly infiltrated after the injection. The temperature reaction of the hog is shown in Chart 2.

June 20. A tender, large edema was present in the submaxillary space, extending along the left parotid region and spreading over the left side of the neck. The animal was less active.

June 21. The swelling had not spread; the hog was lifeless, depressed, refused to eat.

June 22. Edema smaller; the hog was eating a small amount.

June 23. Edema nearly disappeared; the animal active and eating well.

June 26. Edema had disappeared; the hog apparently well.

July 8. A large abscess in the left retropharyngeal region had ruptured overnight. The creamy pus contained clumps of necrotic tissue. The abscess cavity communicated with the left tonsil by a small, fistulous canal. The pus contained cocci, *B. suis*, and spore-bearing organisms resembling the one inoculated. A pure culture could not be obtained. On left tonsil, a small necrotic area was present.

The inoculation of 1.5 c.c. of a broth culture into a tonsil reproduced the clinical picture characteristic of the hogs which were infected during the out-

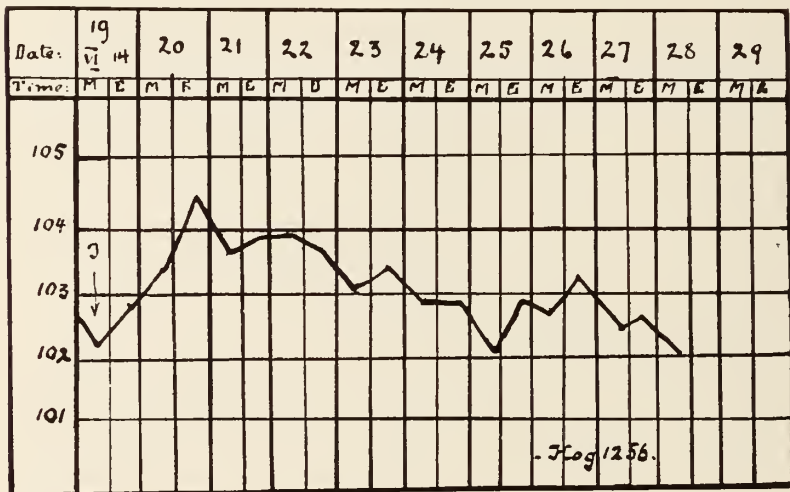


Chart 2.—Temperature curve for Hog 1256.

break, as described. Doubtless the amount inoculated and the virulence were responsible for the fact that the hog did not succumb to the infection. The pharyngeal edema resulted in necrosis of the mucous membrane, ulcer formation in the tonsils, and secondary infection with pyogenic organisms, which gave rise to an abscess in the retropharyngeal region. Microscopically, the inoculated organism was present; a pure culture, however, could not be obtained. Another attempt was made with a large amount of culture.

Hog 4.—Weight, 4,280 grams. Inoculated under ether narcosis, July 8, 1914. in both tonsils, with 3 c.c. to each of a blood broth culture (15 days old).

July 9. The animal was depressed, refused to eat. Temperature 39.10 C.

July 10. Temperature 40 C. A soft, tender swelling in submaxillary space.

July 11. Temperature 39.5 C. The hog breathing with difficulty, with a snoring sound on inspiration and expiration. The mucous membranes slightly cyanosed. The hog refused to eat and was apparently very sick.

July 12 and 13. The swelling was less marked, the breathing less difficult; the hog was eating a small amount.

July 14 to 20. No clinical observations made.

July 21. Hog found dead.

Autopsy: Rigor mortis, slight. Weight, 3,650 grams. The lymph-nodes of the groin were enlarged, appearing hyperemic; the axillary lymph-nodes not enlarged. The regional lymph-nodes in the sublingual spaces were enlarged, the ones on the right being the most marked. These felt firm; on section they appeared whitish; no evidence of suppuration. The submaxillary glands appeared normal. Those on the left-side were enlarged; appeared light on section. The left submaxillary lymph-node appeared normal. The tonsils were represented by a mass of necrotic tissue with two tubular fistulas, one of which communicated with the esophagus. One fistulous tract also extended anteriorly beneath the tongue. The tongue appeared normal. The entire pillars of the fauces presented a necrotic area, about 4 mm. in diameter, yellow in color.

The lymph-nodes of the anterior mediastinum were enlarged, firm, and, on section, white in color, tho presenting a number of small, white-yellowish areas, suggesting necrosis. The peribronchial lymph-nodes were enlarged but showed no caseation. The right lung was adherent to the pleura by numerous recent strips of fibrin. The right lung appeared rather dark red in color. The pleural surface presented numerous white areas, for the most part, circular or ovoid in shape, about 0.5 cm. in diameter. These did not extend into the parenchyma of the lung. On section, the lung exuded a bloody fluid; no areas of consolidation. The left lung was normal in color, with consolidation throughout; numerous small, white areas on surface. The inferior edge of the pleura of the lower lobe presented a mass of fibrin. On section the exudate was bloody in nature. The cut surface presented no pneumonic areas. The heart was normal in size. The pericardium was firmly adherent to the heart. The myocardium appeared pale and anemic.

The abdominal cavity had no excessive fluid. The spleen was slightly enlarged and dark in color, on section showing an increase of connective tissue. The surface presented a number of white, circumscribed areas about 1 cm. in diameter, which did not, however, extend into the substance. On section the surface appeared rather pale, otherwise negative. The kidneys were normal in size but pale; capsule stripped easily; on section appeared anemic, otherwise negative. Both had the same appearance. The mesenteric lymph-nodes were enlarged, on section showing white areas. The intestines contained a very small amount of material.

Diagnosis.—Necrosis of both tonsils; suppuration and necrosis of the pillars of the fauces; lymphadenitis of the retropharyngeal and submaxillary lymph-nodes; fibrinous pleurisy and aspiration-pneumonia of the lobes of the left and right lungs; septicemia.

A very superficial bacteriologic examination revealed *B. coli*, *B. suipestifer*, and bipolar organisms in the heart blood; *B. necrophorus*, and probably the injected organism, and bipolar organisms in tonsils and lung.

The clinical picture was aggravated; the temperature curve was typical, but a detailed study of the symptoms was not made. Doubtless the animal succumbed, not to the infection with the bacillus, but to a septicemia which followed on aspiration-pneumonia. The seat of injection was so severely necrotic and so invaded by secondary organisms that the pneumonia was quite in accordance.

Both experiments on hogs demonstrated that the bacillus isolated from the muscle of the hogs is capable of producing, after intraton-sillary injections, a febrile reaction, and a marked edema in the sub-maxillary and retropharyngeal spaces, which causes disturbances in respiration and swallowing of food. The symptoms are similar to those seen in one of the hogs spontaneously infected. The naturally infected hogs possessed a reduced vitality on account of having been subjected to the hog-cholera immunization, a point which does not enter into the described experiments; furthermore, the virulence and the age of the cultures used are very important. Considering all these points and the transmission experiments with muscle material, we can accept the results as evidence that the bacillus is pathogenic for hogs and most probably enters the system by way of the tonsils or upper digestive tract.

Discussion of pathogenicity tests on animals.—In old text-books and even in some scientific articles, the animal experiment is heralded as one of the best tests of whether an anaerobic organism producing a blackleg-like affection in animals, is *B. chauvæi*, or not. The susceptibility of the various animals is used for the differential diagnosis. Pigeons are absolutely immune to the bacillus of symptomatic anthrax. Lehmann and Neumann,²¹ Günther,²² Gutzeit,²³ and others consider rabbits, rats, and pigeons immune to blackleg infections. Nocard and Roux,²⁴ Leclainche and Vallée,²⁵ Kitt,²⁶ and Foth¹⁸ attribute to rabbits a certain degree of susceptibility. These statements have warranted numerous experiments with many blackleg strains in comparison with the bacillus isolated from the hogs. Some of these tests have been reported elsewhere. The results have not been uniform, neither with the muscle material from blackleg cases nor with our hog culture. The high resistance of rabbits against blackleg material is only relative and doubtless more marked with muscle material than with pure cultures. Exceptions have been noticed however, and it was particularly striking that the bacillus under discussion behaved like one of those rabbit-pathogenic blackleg strains. As in blackleg, large doses only are fatal for rabbits and rats, as is seen from the series of inoculation experiments, reported in Table 6. Any conclusions drawn from

21. Atlas & Grundriss der Bakteriologie, 1912, II, p. 497.

22. Lehrb. der Bakteriologie, 1906, p. 433.

23. Centralbl. f. Bakteriöl., R., 1904, 34, p. 195.

24. Ann. d. l'Inst. Pasteur, 1900, 14, p. 596.

25. Ibid., p. 207.

26. Kolle and Wassermann's Handb. d. pathogen. Microorganismen, 1912, 4, p. 820.

such tests would be entirely erroneous and useless without the necessary microscopic examination and anatomic studies.

Von Hibler²⁷ demands, as prerequisites for pathogenicity tests and the use of the same for differential decisions, that the organisms to be tested should not be in a state of attenuation or atavistic metamorphosis. No doubt every careful bacteriologist will aim to have the organisms which he is testing restored to their original virulence and selective pathogenicity. The means suggested by v. Hibler for achieving such a condition, namely, cultivation in brain, serum, or tissue media, or the procedure of mixed infection, are all satisfactory for some cases, but in my study they have not proved to be very suc-

TABLE 6
THE INFLUENCE OF THE DOSAGE OF CULTURE ON THE MORTALITY

Animal	1.0 c.c.	0.5 c.c.	0.3 c.c.	0.2 c.c.	0.1 c.c.	0.05 c.c.
Guinea-pig	1 injected Died	1 injected Died	1 injected Died	2 injected Died	4 injected Died	
Rabbit.....	2 injected 1 died	3 injected 2 died				
Rat.....	3 injected 1 died	1 injected Lived	1 injected Lived (Died from intercurrent disease)	
Mouse.....	1 injected Died	1 injected Died
Pigeon.....	1 injected Died					

cessful. The virulence once lost could not be restored, and therefore the results on animals are not particularly useful for diagnostic purposes.

On the other hand, the microscopic findings on guinea-pigs, rats, etc., permitted conclusive decisions. It has been shown by Leclainche and Vallée,²⁵ Foth,²⁸ and others that *B. chauvæi* never produces aggregations of filaments on the surfaces of the liver and the peritoneum, a characteristic in common for many organisms belonging to the malignant edema group. The bacillus isolated from hogs is therefore not *B. chauvæi*, but a representative of the malignant edema group.

The character of the lesions in the injected animals, particularly the serous-exudative infiltration of subcutis, is worth consideration

27. Untersuchungen über die pathogenen Anaeroben, etc., 1908, p. 239.

28. Ztschr. f. Infektions-krankh. d. Haustiere, 1909, 6, p. 221; 1910, 8, p. 156.

when studying such closely allied organisms. In blackleg infections, the exudate is always markedly hemorrhagic; in malignant edema infections, however, it is only serous, with occasional hemorrhages. The experiments described show that the infiltration was mostly of the serous type. Different results, when they were obtained, are to be explained, I think, by the fact that large doses, and probably toxins also, were inoculated. These toxins produce hemorrhages with comparative ease, as will be shown later. The local necrosis and the production of gas are inconstant and depend on factors which are not easily controlled.

From the animal experiments one can therefore conclude that the bacillus isolated from hogs is a representative of the malignant edema group; in many respects, however, it is closely related to the *B. chauvæi*.

IMMUNOLOGIC TESTS

Leclainche and Vallée,²⁵ Foth,¹⁸ and Markoff²⁰ suggested the agglutination tests as the ideal method for the identification of anaerobes. The use of guinea-pigs immunized against an identified strain of the suspected organism to be tested, has also been suggested. The latter method, however, is exceedingly delicate and only promising when sufficiently controlled. Either actively or passively immunized guinea-pigs can be used. The writer has not been very successful with the active immunization, and will report on these tests later, when they have been extended along different lines. The agglutination and serum immunization gave striking results, and are here reported more fully:

Agglutination Tests.—The preparation of immune sera offered some difficulties; some of the rabbits lost rapidly in weight and developed subcutaneous abscesses containing *B. cuniculisepticus*. Twenty-four-hour-old, or even twelve-day-old broth or blood-broth cultures, poor in toxins, were injected intravenously at intervals of 7 days. This method of immunization produced, after 4 injections, a very potent serum, as here reported.

RABBIT T.-3.—Immunized with the bacillus of hog disease as follows:

May 18.—0.5 c.c. blood-culture, 24 hours old, intravenously. Weight, 3,100 gm.

May 25.—0.5 c.c. blood-culture, 12 days old (a toxic strain), intravenously. Weight, 3,000 gm.

June 4.—1.0 c.c. broth culture, 6 days old (a toxic strain), intravenously. Weight, 3,200 gm. Bled; agglutinates 1:4,000.

June 15.—3.0 c.c. blood-broth, 8 days old, intravenously. Weight, 3,400 gm.

June 23.—Bled to death. Weight, 2,650 gm. Agglutinates 1:20,000 in 20 minutes.

For comparison, other sera were produced by immunizing rabbits with *B. chauvæi*, "vibron septique," and malignant edema organisms obtained from the American Museum of Natural History in New York.

The bacterial suspensions were either 24-hour-old glucose broth cultures or salt suspensions of centrifugated and washed blood cultures. In some

instances, also, surface cultures on serum agar were suspended in carbolized salt solution. For the tests, fresh cultures only were used; they were shaken, filtered, and standardized to a density equal to a 24-hour-old typhoid broth culture. Dilutions of the serum were prepared as usual by adding 0.1 to 0.0001 c.c., etc., of serum to 1 c.c. of bacterial suspension. The results are shown in Table 7.

This test showed conclusively and more precisely than any other test that the bacillus isolated from hogs is not a *B. chauvæi* nor a malignant edema bacillus (Koch). The agglutination was in every test marked; flocculation occurred in from 5 to 10 minutes and was usually completed in 1 hour. The fact that the "vibron septique" obtained from the Institut Pasteur was agglutinated nearly to the titer limit, appeared first as evidence that the organism isolated from the

TABLE 7
SERUM OF RABBIT IMMUNIZED AGAINST THE HOG BACILLUS

Strain of Organism	Dilution of Serum Causing Complete Agglutination After 24 Hours
Bacillus isolated from hogs.....	1 : 20000
"Vibron septique" de Pasteur.....	1 : 10000
<i>B. chauvæi</i> (Inst. Pasteur).....	1 : 60
<i>B. chauvæi</i> (Kitt, Munich).....	1 : 100
<i>B. chauvæi</i> (California I).....	1 : 40
<i>B. chauvæi</i> (California II).....	1 : 80
<i>B. chauvæi</i> (California III).....	1 : 40
<i>B. chauvæi</i> (Mohler IV).....	1 : 20
<i>B. œdematis maligni</i> . (Koch I).....	1 : 10
<i>B. œdematis maligni</i> . (Koch II).....	—
Controls	—

hogs was a "vibron septique." Comparative tests, which will be reported and discussed elsewhere, proved the identity of the two organisms. The importance of this fact will be the subject of further consideration.

For the identification of anaerobes, the agglutination test seems to be very reliable. I have therefore undertaken a more detailed study of the whole problem and hope to report further on this subject. The complement-fixation test will also be considered at the same time.

Serum-Immunization of Guinea-Pigs.—Foth,¹⁸ Markoff,²⁹ and Wulff³⁰ particularly, demonstrated that old guinea-pigs can be immunized passively by using large amounts of immune serum and that such animals can be used for differential purposes. A few experiments along these lines were carried out further to reinforce the conception that the bacillus isolated from the hogs is not a *B. chauvæi*, but an organism identical probably with the "vibron septique."

30. Deutsch. tierarztl. Wchnschr., 1912, 20, p. 611.

The guinea-pigs were injected with serum and simultaneously with the culture (24-hour blood broth) to be tested. Some of the results are tabulated in Table 8.

These experiments demonstrated that the rabbit immune serum obtained with the hog bacillus, protected the guinea-pigs against the bacillus and against the "vibrion septique" of the Institut Pasteur. Animals immunized against the hog bacillus were tested 17 days afterwards and proved to be immune only to the "vibrion septique," but not to any blackleg strain.

The conclusion can therefore be reached that serologically the bacillus isolated is related only to the "vibrion septique," and is positively not a *B. chauvæi*.

TOXIN PRODUCTION BY THE BACILLUS

Sterile filtrates of 4- to 10-day-old glucose broth cultures, prepared and treated in the essential points like the blackleg toxin of Grassberger and Schatzenfroh,³¹ contained toxins which proved fatal to rabbits, guinea-pigs, and white mice by intravenous or intraperitoneal inoculations. The very few tests carried out do not permit of final conclusions, and a detailed report is postponed until some essentially important and necessary comparative tests have been completed.

GENERAL DISCUSSION WITH REFERENCE TO THE IDENTIFICATION OF THE BACILLUS CAUSING A BLACKLEG-LIKE DISEASE IN SWINE

From the muscles of two hogs which had succumbed to a disease resembling blackleg in the gaseous, hemorrhagic condition of the muscles, an anaerobic bacillus was isolated. A detailed bacteriologic examination was carried out with a view to throwing more light on the question of so-called "blackleg" in swine and of determining whether such infections—when most similar anatomically—are actually due to *B. chauvæi*. The problem undoubtedly presents numerous questions concerning anaerobic organisms in general. Therefore, it is not surprising that the study of the bacillus developed into a comparison of the well-known pathogenic anaerobes of the blackleg-gasphlegmon malignant edema group. Even tho the report does not specifically mention all these detailed investigations, nevertheless they had to be carried out, and the entire aspect of the study was therefore broadened.

It is quite evident that the organism isolated has certain features in common with the malignant edema bacillus, particularly with the

31. Handbuch der Technik und Methodik der Immunitätsforschung, 1907-1908, 1, p. 161.

TABLE 8
EFFECT OF ANTIHOG-DISEASE SERUM ON GUINEA-PIGS

Weight of Guinea-pigs in Grams	Date of Inoculation	Amount of Serum in c.c.	Test Cultures	Amount of Culture in c.c.	Result	Control Test on	Culture	Result
720	May 29, 1914	5.0	Hog bacillus	0.25	June 16, 1914	Blackleg (Mun-ich) 0.1	Died in 22 hr.
735	May 29, 1914	..	Hog bacillus	0.1
520	May 29, 1914	5.0	Hog bacillus	0.2	Died in 32 hr.	June 16, 1914	"Vibron septique" 0.2
664	May 29, 1914	5.0	Hog bacillus	0.25	June 16, 1914	Hog bacillus 0.1
600	May 29, 1914	4.0	"Vibron septique"	0.25	June 16, 1914	Blackleg (In s t. Pasteur) 0.2	Dead in 18 hr.
470	May 29, 1914	4.0	"Vibron septique"	0.25	June 16, 1914	"Vibron septique"
676	June 16, 1914	4.0	Blackleg (Calif.)	0.2	Died in 24 hr.
538	June 16, 1914	5.0	Blackleg (Mun-ich)	0.25	Died in 18 hr.
821	June 16, 1914	6.0	Blackleg (P a s -teur)	0.25	Died in 30 hr.

"vibron septique" of the Institut Pasteur, of which an original culture served as control for my experiments. One would be justified in diagnosing the isolated organism as a "vibron septique," and, inasmuch as Koch³² considered this organism identical with the bacillus which he and Gaffky³³ had obtained from soil, the bacillus of the hog muscle would be a bacillus of malignant edema. The latter deduction is proved wrong however by several differences in my observations from the descriptions given by Koch and others.

The original descriptions³⁴ of the "vibron septique" and *B. œdematis maligni* are so incomplete that, based on the meager data, they could not be considered, today, as being identical, since the perfected bacteriologic technic has shown that anaerobiosis, motility, sporulation, microscopic appearance, and animal pathogenicity are insufficient criteria for an identification. All investigators (Liborius,³⁵ Jensen and Sand,³⁶ Kitasato,³⁷ Carl,³⁸ Silberschmidt,³⁹ etc.) who identified anaerobes which they had isolated from various sources with the malignant edema bacillus of Koch, state that the bacillus peptonizes the coagulated milk and produces in this medium a very disagreeable odor. On the other hand, the statements regarding the "vibron septique" are incomplete. Arloing⁴⁰ mentions that the virus of septicemic gangrene ferments carbohydrates and albuminoids; Besson,⁴¹ on the other hand, states in his text-book that the "vibron septique" peptonizes coagulated egg albumen, serum, etc. The organism described by the writer never showed peptonization in any medium. The study of the literature gave the impression at first that the bacillus isolated from the hogs represented a new anaerobe. However, by studying carefully the splendid book and identification tables of v. Hibler,⁴² the writer found a proper identification immediately possible. The bacillus corresponds in every detail with the so-called Ghon-Sachs bacillus.⁴³

The Ghon-Sachs bacillus does not blacken the brain medium, or change its reaction to alkaline; the malignant edema bacillus, however,

32. Mitt. a. d. k. Gsndhtsamte, 1881, 1, p. 49.

33. Ibid., p. 93.

34. Bull. de l'Acad. de méd., 1877, 6, p. 781. Compt. rend. Acad. d. sc., 1878, 86, p. 1037. Bull. de l'Acad. de méd., 1881, 10, pp. 78, 81, 142.

35. Ztschr. f. Hyg. u. Infektionskrankh., 1886, 1, p. 158.

36. Deutsch. Ztschr. f. Tiermedizin, 1888, 13, p. 41.

37. Ztschr. f. Hyg. u. Infektionskrankh., 1889, 6, p. 107.

38. Deutsch. tierarztl. Wehnschr., 1895, 5, p. 115.

39. Korrespondenzbl. f. Schweizerärzte, 1900, 30, p. 361.

40. Leçons sur la tuberculose et certaines septicémies, 1892.

41. Technique microbologique et sérothérapique, 1911, p. 288. Untersuchungen über die pathogenen Anaeroben, etc., 1908.

42. Berichte d. naturwissenschaftl. medizinisch. Vereins in Innsbruck, 1908-1909.

43. Centralbl. f. Bakteriöl., I, O., 1903, 34, p. 301; 1904, 35, p. 655; 1904, 36, p. 1.

does. The milk when coagulated is not peptonized, and remains acid when inoculated with the Ghon-Sachs bacillus. The spores are resistant to a temperature of 99 C. for 8 minutes only. On the other hand, the spores of the malignant edema bacillus resist this temperature for more than 60 minutes. Furthermore, the serologic tests permit a distinct separation of these organisms.

The description given by Ghon and Sachs⁴³ of the bacillus isolated by them in 1901 and reported in 1903, corresponds in every respect with the bacillus isolated from our hogs. These two writers have already indicated that their organism was identical with the "vibron septique"; but, inasmuch as no complete description of the true malignant edema bacillus (Koch) was on hand, the bacillus isolated by Ghon and Sachs, the "vibron septique," and the malignant edema bacillus were thrown together and identified as one and the same bacterium.

Through the careful comparative study of v. Hibler,⁴⁴ a separation of the organisms has now been accomplished, and any future work should first be compared with his very detailed description of the various pathogenic anaerobes.

The Ghon-Sachs bacillus has been recognized and properly identified 7 times in diseased conditions, all of which resembled, clinically, malignant edema or gas-phlegmon—1 case in man reported by Ghon-Sachs,⁴³ 4 cases by v. Hibler,⁴⁴ 2 in cattle (v. Hibler⁴⁵), and 1 in a horse (Schlemmer⁴⁶).

In animal pathology it unquestionably plays a considerable rôle. The various descriptions given by Miessner,⁴⁷ Markoff,²⁹ Levens,⁴⁸ and others for anaerobic organisms responsible for parturient symptomatic anthrax, bradsot of sheep, and blackleg of horses, correspond in almost every point with the facts brought forward by this study. Most of the workers, however, did not extend their studies sufficiently far to enable them to identify the organisms with the Ghon-Sachs bacillus.

The organisms isolated from hogs by Mareck,¹ even tho morphologically identical with *B. chauvæi*, had a high pathogenicity for rabbits; the publications of Mareck do not contain any detailed account as to the biochemical actions of the observed organism. The same remarks apply to the publications of Born² and Battistini³ in which,

44. Untersuchungen über die pathogenen Anaeroben, etc., 1908, p. 2.

45. *Ibid.*, 1, p. 404, p. 405.

46. Berl. tierärztl. Wehnschr., 1913, 19, p. 905.

47. Mitt. a. d. k. Wilhelm-Inst. f. Landwirtsch. in Bromberg, 1909, 1, p. 28.

48. Berl. tierärztl. Wehnschr., 1911, 17, p. 413, p. 673.

based purely on morphologic similarity of the observed organisms in the diseased muscles, the diagnosis of symptomatic anthrax in hogs was made. Kitt⁴⁹ observed blackleg-like lesions in several wild hogs and, as a causative agent, he stated that he found the bacillus of malignant edema. Von Hibler isolated the "Novy bacillus" from the dried muscle of a wild hog which had succumbed to a blackleg-like infection following a lacerated wound on the hind leg.

The study of the anaerobe had already been completed when, in September, 1914, I found a reference to a publication by Köves⁵⁰ in which the same disease in hogs under discussion in this paper had been investigated on about 15 hogs. Later, I found that in February, 1914, Köves⁵¹ summarized, in a preliminary note, his work on so-called "Rauschbrand der Schweine," stating that he found the Ghon-Sachs bacillus to be responsible for the affection. Furthermore, he mentions that in some cases of extensive diphtheric gastritis in hogs, which has always been considered as typical for hog-cholera, he isolated from the serosa of the stomach the Ghon-Sachs bacillus. The identity of the organism was proved by animal inoculations; the organism isolated from the serosa of the stomach also produced typical muscle lesions with gas formation. The conclusion that the so-called "symptomatic anthrax" of swine and the bradsot-like affections of these animals are etiologically an entity, is now fully proved by my independent investigations.

In the introduction to this paper I stated that "symptomatic anthrax" in swine had not been observed in the United States; the same statement also includes infections due to the Ghon-Sachs bacillus. All the strains obtained from the American Museum of Natural History behaved like the *B. oedematis maligni* (Koch) and a careful review of the existing literature indicates that all organisms isolated from cases of malignant edema peptonize the milk and coagulate proteins. How carefully such studies have been carried out is not always evident. One statement, made by Sperry and Rettger,⁵² that the bacillus of symptomatic anthrax causes rapid decomposition of fibrin, egg, and meat mixtures, and reduction of solid matter, suggests in the light of all of the comparative work on *B. chauvæi* published in the international literature, that the strains used in some laboratories

49. Bakterienkunde und pathologische Mikroskopie, 1908, p. 290.

50. Deutsch. tierärztl. Wchnschr., 1914, 22, p. 536.

51. Berl. tierärztl. Wchnschr., 1914, 30, p. 134.

52. Jour. Biol. Chem., 1915, 20, p. 455; 1906-07, 2, p. 79.

have not always been properly identified. It would be very interesting if careful data as to the frequency and occurrence of the Ghon-Sachs bacillus in the United States were collected.

In a recent report on studies on hog-cholera, Dinwiddie⁵³ describes lesions in the stomach of hogs, and an anaerobic organism, the first being similar to those noted and described by Köves, the latter probably identical with the strains isolated by myself and therefore belonging to the Ghon-Sachs bacillus type. The intestinal lesions, in form of a severe diphtheric gastritis described by Köves as being caused by the Ghon-Sachs bacillus, are very frequently seen and are unquestionably mistaken for hog-cholera. An extensive investigation, guided by these stated facts, would therefore be a very gratifying task.

The organism seems to be widely distributed, not confined to Europe and America only; a description given from Australia by Gilruth⁵⁴ on a variety of the "vibrion septique" non-pathogenic for rabbits corresponds in many respects with the Ghon-Sachs bacillus.

The discussion cannot be closed without considering the experiments by v. Ratz⁵ regarding the transmissibility of symptomatic anthrax to hogs. In 9 experiments, in which primary muscle-juice of cattle or hogs was used, v. Ratz succeeded in infecting 9 animals, of which 3 died. Experiment 2 consisted in the inoculation of a hog with muscle juice from so-called "symptomatic anthrax" in a hog. The lesions resembled strongly those described in this paper and, in the writer's mind, there is no doubt that v. Ratz was working, in this case, with the Ghon-Sachs bacillus. In two other successful experiments (4 and 8), muscle juice from cattle was used, and the changes resembled more those of "symptomatic anthrax." In no instance, however, did v. Ratz carry out cultural tests, nor does he report the methods he used to identify the organism he inoculated with the muscle juice. He injected the material from cattle, supposing that all lesions like symptomatic anthrax are caused by *B. chauvæi*. That his supposition is totally erroneous has been shown by Wulff,³⁰ Foth,¹⁸ and many others. It is therefore natural to conclude that either the muscle material did not contain *B. chauvæi*, but the Ghon-Sachs bacillus; or a mixed infection of *B. chauvæi* with the Ghon-Sachs bacillus existed. In any case, these experiments do not prove that

53. Bull. Arkansas Exper. Station, 117, p. 600.

54. Veterinary Jour., 1911, 67, p. 471.

the hog has only a limited immunity to blackleg and that spontaneous cases are probably due to *B. chauvæi*.

The experiments of Glässner⁶ and Wulff,⁷ who attempted to infect hogs with blackleg material, are more careful. In 2 experiments the writer has inoculated small hogs with muscle material containing a very virulent *B. chauvæi* strain; aside from a slight, local swelling no symptoms were noted. I cannot therefore agree with v. Ratz that hogs are susceptible to symptomatic anthrax. Anatomically similar affections of hogs are probably always caused by the Ghon-Sachs bacillus which, morphologically, is so remarkably similar to *B. chauvæi*.

CONCLUSIONS

The report describes the recognition in the United States of a disease of hogs, well described in European publications, which anatomically resembles symptomatic anthrax in cattle. The cases studied belonged to a small epidemic and are not sporadic cases as reported from Hungary.

The lesions and the deaths of the animals were due to the Ghon-Sachs bacillus, which was properly identified by all well-known means. Etiologically, the American cases are therefore identical with the European ones.

The methods and media recommended by v. Hibler, the agglutination tests and the serum immunization of guinea-pigs, proved to be very reliable for the separation and identification of closely allied anaerobes.

The name of "specific gas phlegmon of hogs" is proposed by the writer for the disease.

The study of this disease, additional experiments, and a critical survey of the literature fail to prove that hogs are spontaneously attacked by symptomatic anthrax, or that they are susceptible to *B. chauvæi*.

NATURAL HEMOLYSINS IN NORMAL HORSE SERUM*

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During the study of the complement-fixation test for glanders in horses, our attention was attracted by the question of a natural hemolysin for the corpuscles of the sheep and the ox.

A search of the literature has failed to give us the desired information. Morgenroth and Sachs¹ state "it is worthy of note that in almost every horse serum which we examined for the purpose, we found a considerable amount of amboceptor for guinea-pig blood. The amboceptor was characterized by a particularly high degree of thermolability, being invariably destroyed by heating to 55 degrees C. A complement for the same is very often absent, and even when present it is only on the addition of considerable amounts of fresh guinea-pig serum that this amboceptor becomes manifest." Sachs,² in his table compiled from the literature, mentions as doubtful the presence of natural amboceptors for the erythrocytes of guinea-pigs and rabbits, and an absence of the same for the erythrocytes of other vertebrates.

Having sufficient material constantly at hand, we decided to determine this point to our own satisfaction. After the examination of a sufficient number of sera for the presence of antishoop and antiox hemolysins had been completed, it was thought of interest to continue the work with the erythrocytes of other vertebrates as follows: guinea-pig, hog, goat, cat, white mouse, man, rabbit, chicken, and dog.

TECHNIC

Serum.—The sera of normal horses used in these experiments were separated from blood collected from the jugular vein into sterile test-tubes, which during the clotting were kept in an upright position in the ice-chest. The clear serum was then removed and inactivated by heating to 60 C. for one-half hour to destroy the anti-complementary substances and to render inactive any native complement. At first the amounts of 0.4 c.c., 0.3 c.c., 0.2 c.c., 0.1 c.c., and 0.5 c.c. were employed. Later, after having observed the continued negative results obtained with sheep corpuscles, we abandoned the last four dilutions, using only

* Received for publication July 1, 1915.

1. Ehrlich: Collected Studies in Immunity, 1906.

2. Kolle and Wasserman's Handb. d. pathogen. Microorganismen, 1913, 2,² p. 793.

one tube for each serum in the dose of 0.4 c.c., with the intention of retesting in higher dilutions should any show traces of hemolysis with this amount.

Complement.—Fresh guinea-pig serum was employed as a complement in the dose of 1 c.c. of a 5% normal salt solution. In each case the complement was tested for the presence of native amboceptors for the erythrocytes to be tested, and it was found that guinea-pig serum frequently possessed sufficient antidog hemolysin to produce traces of hemolysis when used in the dose of 1 c.c. of a 1:20 dilution. However, we have not classed this in the category of amboceptors for the reason that treatment with analogous cells failed to remove the hemolysin. Amboceptors for the various other cells tested were not sufficient to produce any hemolysis when used in this dilution.

Erythrocytes.—With the exception of that from sheep corpuscles, the corpuscle suspensions were always prepared from fresh blood and used within 24 hours from the time collected, after thorough washing. The sheep cells used varied in age from 1 to 8 days, the corpuscles of but one sheep being used. The blood of this animal had previously been tested for its keeping properties and was found to keep from 2 to 3 weeks without becoming unduly fragile if preserved in its own serum and washed only as needed. In all the tests a dose of 1 c.c. of a 25% suspension of washed corpuscles was used.

TABLE 1
EXAMINATION OF HORSE SERA FOR NATIVE ANTISHEEP HEMOLYSIN

Amount of Serum; c.c.	100% Hemolysis	75% Hemolysis	50% Hemolysis	25% Hemolysis	0 Hemolysis
0.4.....	3	0	1	0	196
0.3.....	0	1	1	2	196
0.2.....	0	0	0	2	198
0.1.....	0	0	0	0	200
0.05.....	0	0	0	0	200

Salt Solution.—Enough salt solution (0.85%) was used in each tube to make a total volume of approximately 3 c.c. Control: The maximal dose of serum was tested with the erythrocytes used to determine whether inactivation was complete. These sera were also tested with the anti sheep hemolytic system to make sure that they were not anti-complementary. The usual dose of complement was tested with the erythrocytes to make certain that all the native amboceptors were removed. The corpuscle suspension was controlled in normal salt solution for undue fragility. Readings: All readings were made at the end of 2 hours' incubation at 37 C. The control tubes, however, were placed in the ice-chest and examined on the following morning.

Antisheep Hemolysins.—The results of the examination of 200 horse sera for native antisheep hemolysin are shown in Table 1. Of the 200 sera tested, but 4 gave any trace of hemolysis. Of these, 3 gave complete, and one 50% hemolysis in dilutions of 0.4 c.c. The remaining 196 were completely negative. Converting the figures which represent the number of sera into percentages, we find but 1.5% capable of producing complete hemolysis with 0.4 c.c. of serum, and 98% entirely negative as regards any signs of hemolysis.

For fear that the animals the sera of which were positive in the test for amboceptors might possibly be suffering from some disease, a careful clinical examination was made, which showed them to be in perfect physical condition.

Antidog Hemolysin.—During the study of these cells we encountered the presence of natural hemolysin in guinea-pig serum used as complement. The failure to remove it by the usual methods employed, led us to a series of experiments with these cells which resulted in finding them subject to a very peculiar hemolysis. For example, guinea-pig serum, as well as horse serum, was found capable of producing hemolysis of these corpuscles without the activity of a complement. A more detailed report of these experiments will be published later.

Examination of other Bloods.—The cells of other vertebrates were tested with one dose of serum, namely, 0.4 c.c. All the essential data, including the various bloods employed, the number of sera tested, and the results obtained with this amount, have been summarized in Table 2.

TABLE 2
SUMMARY OF NATURAL HEMOLYSINS IN HORSE SERUM (0.4 c.c. AMOUNTS)

Blood	Number of Sera Tested	Percentage Showing 100% Hemolysis
Sheep	200	1.5
Ox	100	0
Guinea-pig	50	0
Hog	50	0
Goat	50	0
Cat	50	0
Mouse (white)	50	0
Rabbit	50	0
Chicken	50	0
Man	50	0

CONCLUSIONS

Of 200 sera examined for antisheep hemolysin, 1.5% were found capable of producing complete destruction of these cells in the amount of 0.4 c.c.

Horse serum was found to possess the property of dissolving the red cells of the dog without the aid of complement.

Native hemolysins for the cells of guinea-pig, hog, goat, cat, white mouse, rabbit, chicken, and man were not found.

THE EFFECT OF BACTERIAL METABOLISM ON THE ANTIGENIC PROPERTIES AND SEROLOGIC REACTIONS OF BACTERIA *

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The specific effect of the presence of fermentable sugar upon bacterial metabolism, as shown by Kendall and Farmer,¹ and its equally definite effect upon the production of proteolytic ferments by *B. proteus*, as shown by Kendall and Walker,² made it a matter of interest and perhaps of practical importance to determine whether growth in media containing utilizable carbohydrate would affect the antigenic properties of bacteria, or would in any way influence their reaction to normal and immune sera.

To this end, tubes of sugar-free broth and of 1% dextrose broth were inoculated from the same stock culture of *B. typhosus*. Daily transfers were made into corresponding media for a period of 2 weeks. At the end of this time cultures were made on sugar-free and dextrose agar slants, incubated for 24 hours, and then placed in the refrigerator (5 to 10 C.) for preservation. The sugar-free culture was designated "T. P.," the dextrose culture "T. D."

Two observations may be noted incidentally: (1) The growth of "T. D." was very much more abundant in dextrose media than that of "T. P." on sugar-free media—an observation which is not by any means new. (2) There was a definite tendency for "T. D." to die out rather quickly at first, whether the cultures were kept in the refrigerator or at room temperature, so that it was necessary to make fresh inoculations at least once a week. After cultivation for from 4 to 6 months on dextrose media, growth could easily be obtained by inoculation from the sediment of dextrose broth cultures from 3 to 4 weeks old.

To determine the effect of sugar on the antigenic properties of *B. typhosus*, healthy rabbits were injected intraperitoneally, one with a single large dose of a suspension of "T.D." and one with the same

* Received for publication July 3, 1915.

1. Jour. Biol. Chem., 1912-13, 12, pp. 13, 215, 219.

2. Tr. Chicago Path. Soc., 1915, 9, p. 320.

of "T. P.," heated to 56 C. for 30 minutes. On the tenth day thereafter the animals were bled and the serum obtained. The antibody content of these sera for the homologous and heterologous strains was determined.

AGGLUTININS

Numerous attempts have been made to modify the agglutinogenic and agglutinative properties of bacteria.

Kirstein³ found that typhoid bacilli when grown on urine agar were more readily agglutinated, and when grown in serum broth slightly less readily agglutinated, than when grown in ordinary media. He was unable to produce any permanent loss of agglutinability in the strains studied by him. Gloessner⁴ reported that the presence of sugar in culture media had an unfavorable influence on the agglutinogenic properties of *B. typhosus* but had little or no effect upon agglutinability. Sehrwald⁵ claimed that *B. typhosus* grown upon potato was more readily and more rapidly agglutinated than when cultivated on any other medium. Eisenberg⁶ was unable to confirm this finding of Sehrwald's. Eisenberg grew *B. typhosus* for several generations in broth at 42 C. and found a reduction in agglutinative, but not in agglutinogenic, properties.

Porges and Prantschoff⁷ injected a suspension of living typhoid bacilli into the ear vein of a rabbit, and 35 days later isolated the same organism from the gall-bladder of the animal. They did not find that this organism agglutinated at all differently from the original culture which had been kept growing on ordinary media. Altmann and Rauth,⁸ by growing *B. coli* for several generations on "Carbol-agar," produced a strain which would not agglutinate with the immune serum of the original strain, nor would the immune serum of this organism agglutinate the original strain of colon bacillus from which it came. Bordet and Sleswijk⁹ grew the bacillus of pertussis on plain and hemoglobin agar. The serum of rabbits inoculated with the plain agar strain would agglutinate only that strain; while the serum of rabbits inoculated with organisms grown on hemoglobin agar would agglutinate both plain and hemoglobin agar strains.

Theoretically, there are two possible ways in which differences in metabolism of bacteria due to the presence of fermentable sugar may affect agglutination. The agglutinogenic substance may be reduced or modified so that injection of the organisms into animals will cause the production of no agglutinins, or of a serum of low titer only. Or the agglutinable substance may be diminished or changed so that even in the presence of sera of high titer the organisms will not agglutinate. It appears from the literature that it is very difficult to affect changes of either kind in a given strain of bacteria. Such changes as are pro-

3. Ztschr. f. Hyg. u. Infectious-Krankh., 1904, 46, p. 229.

4. Ztschr., f. exper. Path. u. Therap., 1905, 1, p. 640.

5. Deutsch. Med. Wchnschr., 1905, 31, p. 261.

6. Centralbl. f. Bakteriöl., I, O., 1906, 41, pp. 752, 823.

7. Ibid., p. 658.

8. Ztschr. f. Immunitätsforsch., 1910, 7, p. 629.

9. Ann. de l'Inst. Pasteur, 1910, 24, p. 476.

duced are, as a rule, quantitative rather than qualitative, and transitory rather than permanent. In general, our results accord with this statement. But the presence of utilizable sugar does appear to have a very definite and constant effect upon agglutination, as shown by the results here reported.

TABLE 1
EFFECT OF BACTERIAL METABOLISM UPON THE AGGLUTINOGENIC AND AGGLUTINATIVE PROPERTIES OF *B. TYPHOSUS*

Culture		T. D. Immune Serum								
		1-50	1-100	1-250	1-500	1-750	1-1000	1-2000	1-3000	1-4000
I	{T. D.	+++	+++	+++	+++	+++	+++	+++	++	..
	{T. P.	+++	+++	+++	+++	+++	+	—	—	..
II	{T. D.	+++	+++	+++	+++	+++	+++	++	—	..
	{T. P.	+++	+++	+++	+++	+	—	—	—	..
		T. P. Immune Serum								
I	{T. D.	+++	+++	—	—	—	—	—	—	—
	{T. P.	+++	+++	—	—	—	—	—	—	—
II	{T. D.	+++	+++	+++	++	++	+	—	—	—
	{T. P.	+++	+++	+++	—	—	—	—	—	—

The macroscopic method of agglutination was used throughout this study. Suspensions of 24-hour sugar-free and dextrose agar slant cultures were employed to avoid the effects of differences in acidity in sugar-free and dextrose broth.

Table 1 shows characteristic results from the injection of 2 rabbits with suspensions of "T. D." and of 2 rabbits with correspondingly heavy suspensions of "T. P." From this table it appears (1) that a serum of higher titer is obtained by the injection of suspensions of killed typhoid bacilli of the "T. D." strain than by the injection of suspensions of *B. typhosus* of the "T. P." strain; and (2) that the dextrose strain is more readily agglutinated than the sugar-free strain, even with the heterologous serum. It would seem therefore that the presence of utilizable carbohydrate in media either had increased the amount of both agglutinogenic and agglutinable substances of this strain of typhoid bacilli, or, more likely, its absence had decreased the amount of these substances.

OPSONINS

A comparatively small number of observations were made upon the production of opsonins for "dextrose" and "sugar-free" strains by the injection of rabbits with suspensions of killed cultures of T. P. and T. D., respectively. Table 2 shows a characteristic result,

from which it appears that the "dextrose" strain was more readily engulfed by leukocytes than the "sugar-free" strain, even in the presence of T. P. immune serum. But the percentage of leukocytes engaged in phagocytosis was greater in the presence of the homologous than of the heterologous serum.

TABLE 2
EFFECT OF BACTERIAL METABOLISM UPON THE PHAGOCYTABILITY OF B. TYPHOSUS

Culture	T. D. Immune Serum		T. P. Immune Serum	
	Phagocytic Index	Percentage of Leukocytes Containing Bacilli	Phagocytic Index	Percentage of Leukocytes Containing Bacilli
T. D.	3.7	59	2.6	38
T. P.	1.4	38	1.9	52

LYSINS

An attempt was made to determine whether there was any difference in the lysinogenic properties of T. P. and T. D., and also to discover whether the two strains differed in any way in their susceptibility to bacteriolysis. The technic first described by Neisser¹⁰ was employed. The results obtained by using immune serum were very confusing and lacking in uniformity. The cause of this was found to be a variation in the amount of lysin in different specimens of normal guinea-pig or rabbit serum used as complement, and in the difference in the effect of this normal lysin on the dextrose and sugar-free strains. Hence, it was necessary to limit this study to the investigation of the susceptibility of these two strains to the lysins of normal serum.

The serum was not inactivated and was tested within 3 hours after the blood was drawn. The technic was as follows: Dilutions of 1:10, 1:20, etc., up to 1:100 were made in physiologic salt solution. One cubic centimeter of each dilution was placed in each of 2 test tubes. To each tube of one set of dilutions was added 1 c.c. of a suspension of the dextrose strain, made by inoculating one loopful of a 24-hour dextrose-broth culture into 50 c.c. of dextrose broth. To each tube of the other set there was added 1 c.c. of a suspension of the sugar-free strain, made by inoculating 2 or 3 loopfuls of a 24-hour sugar-free broth culture into 50 c.c. of broth. (The difference in number of loopfuls of culture used in the two instances was due to the difference in density of growth of the two strains in their respective media.) Each set of tubes was incubated for 3 hours. Agar plates were then made by pouring the cooled, melted agar into the mixture of bacillary suspension and serum dilution and thence into Petri dishes. The plates were then incubated for 24 hours. The amount of bacteriolysis was in inverse proportion to the number of colonies on these plates.

10. P. Ehrlich: Collected Studies on Immunity, 1906.

As controls, 1 c.c. of each bacillary suspension was placed in each of 2 test tubes containing 1 c.c. of physiologic salt solution. Agar plates were made immediately, as described, from 1 tube of each diluted suspension. The other two tubes were incubated for 3 hours and plates poured at the end of that time. All four plates were incubated for 24 hours. The first two of the control plates indicated the number of bacilli in each of the suspensions at the beginning of the experiment; the last two showed the number to which these had increased when not hindered by the presence of a lytic serum.

Charts 1, 2, and 3 show the effect of normal guinea-pig, rabbit, and human sera upon the T. P. and T. D. strains. From these charts it is seen that the dextrose strain is very much more easily destroyed by the lytic action of normal sera than is the sugar-free strain. Control tests showed that the reduction in the number of colonies on the plates poured from the serum mixtures was not due to agglutination. As shown in the charts, many of the plates were entirely sterile.

It may be objected that in all but one instance there were more bacilli per cubic centimeter to begin with in the suspension of the sugar-free strain than in the dextrose strain. But the greater rate of growth of T. D. would at least partially balance this. Furthermore, the number of bacilli present after 3 hours' incubation shows such a vast difference in the two sets of tubes that it hardly seems possible to account for the result solely on the basis of a not very great initial difference in the density of the suspensions.

ENDOTOXIN

Theobald Smith long ago called attention to the fact that *B. diptheriae*¹¹ and *B. tetani*¹² do not form toxins in dextrose broth. The following single observation is presented because it suggests that the presence of dextrose in media may influence the toxicity of the endotoxin of *B. typhosus*. Suspensions in physiologic salt solution were made of cultures of T. D. and T. P. grown on dextrose and sugar-free agar hardened in 16-ounce French square bottles. Each suspension consisted of the growth on 3 such bottle-plates of the respective organism. They were made up to as nearly equal density as possible and equal volumes of each taken for further treatment.

To each suspension were added 3 c.c. of homologous immune (rabbit) serum and 2 c.c. of fresh normal rabbit serum for complement. The mixtures were incubated for 6 hours, and then passed through a Pasteur filter. One cubic centimeter of each filtrate inoculated into

11. Tr. Assn. Am. Phys., 1896, 11, p. 37.

12. Cited by Kendall: Jour. Med. Research, 1911, 24, p. 411.

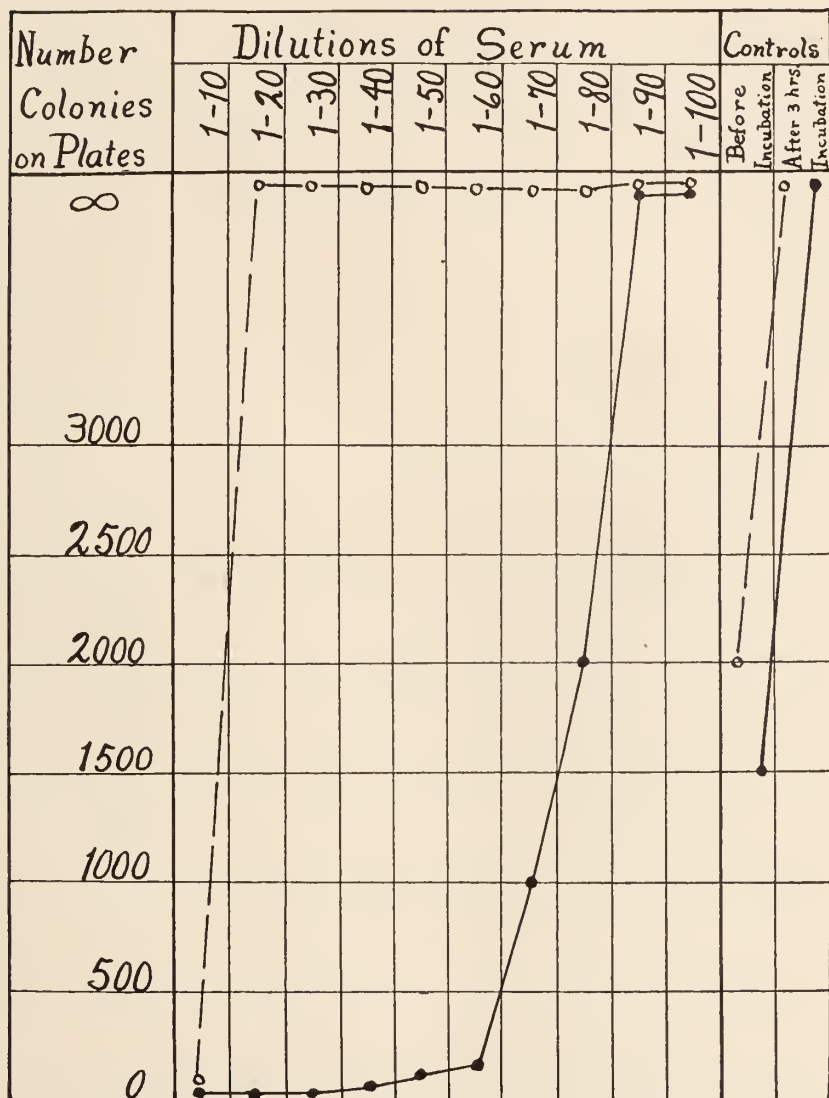


Chart 1.—The difference in susceptibility of T. D. and T. P. strains of *B. typhosus* to bacteriolysis by normal guinea-pig serum. The broken line represents T. P.; the solid line, T. D.

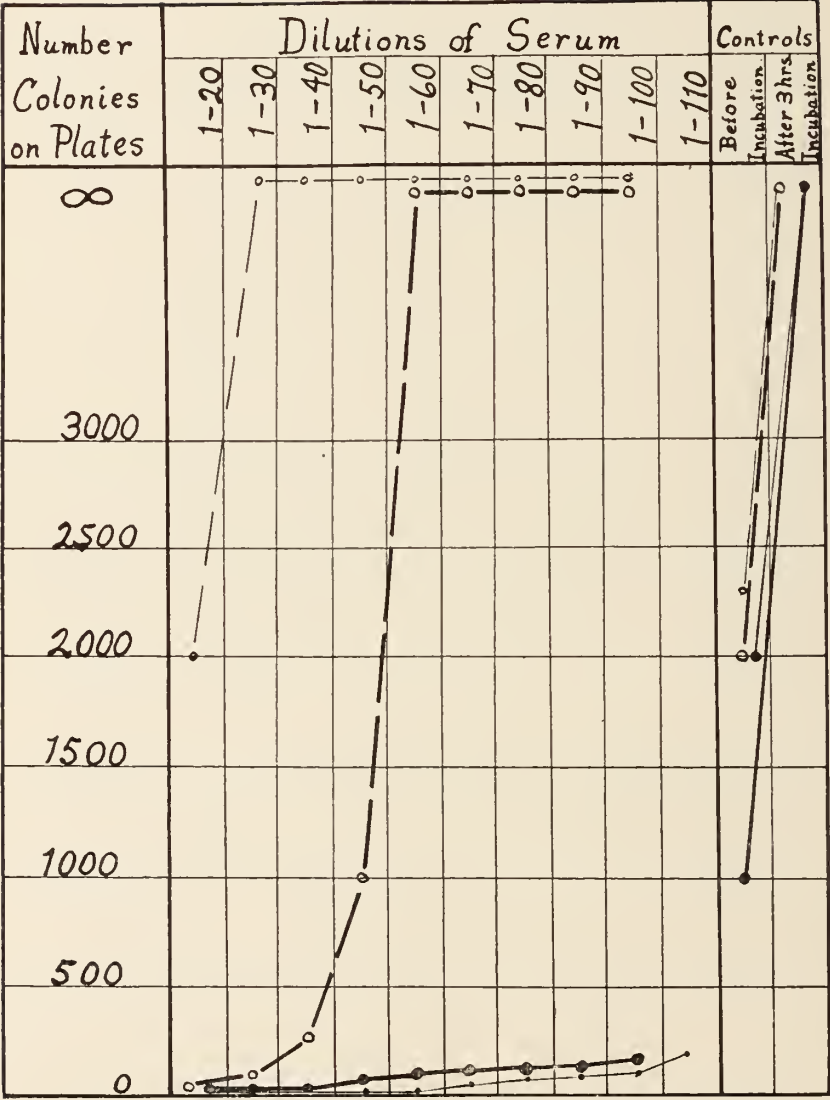


Chart 2.—The difference in susceptibility of T. D. and T. P. strains of *B. typhosus* to bacteriolysis by normal rabbit serum. The broken lines represent T. P. in Experiments 1 and 2. The solid lines represent T. D. in Experiments 1 and 2.

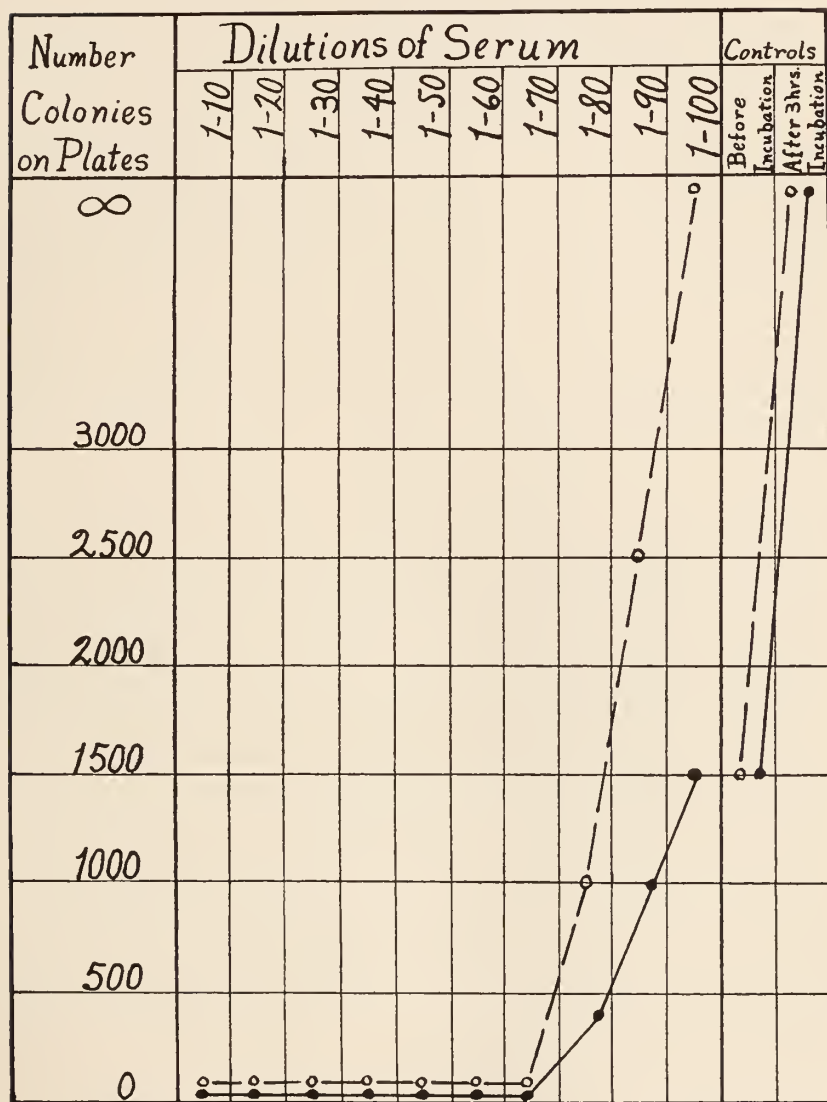


Chart 3.—The difference in susceptibility of T. D. and T. P. strains of *B. typhosus* to bacteriolysis by human serum. The individual from whom the serum was obtained received 3 doses of antityphoid vaccine 3 years previously. The broken line represents T. P.; the solid line, T. D.

broth and incubated showed no growth. Two rabbits of approximately the same weight were injected intravenously with 12 c.c. of the filtrates. The results are shown in the following reports:

RABBIT 10.—WEIGHT 1750 GRAMS

Jan. 29.—11:00 a. m.: Temperature 99.5 F.; 11:35 a. m.: 12 c.c. T. D. filtrate intravenously; 2:00 p. m.: Temperature 101.2 F.

Jan. 30.—11:00 a. m.: Does not appear ill. Temperature 103.8 F. Leukocytes 75,800 (?) per cubic millimeter. Counting very difficult because of unavoidable tendency of leukocytes to form clumps. Polymorphonuclears 89.5%. Lymphocytes 7.5%. Large mononuclears 3.0%. Two nucleated reds seen in counting 500 leukocytes.

Feb. 1.—11:00 a. m.: Temperature 101.2 F. Appears normal.

Feb. 2.—11:00 a. m.: Leukocytes 19,300 per cubic millimeter. Polymorphonuclears 35.0%. Lymphocytes 41.0%. Large mononuclears 16.3%. Myelocytes (?) 2.7%. Basophils 5.0%. Two nucleated reds found in counting 400 leukocytes. Animal appears well.

RABBIT 11.—WEIGHT 1800 GRAMS

Jan. 29.—11:05 a. m.: Temperature 97.4 F.; 11:30 a. m.: 12 c.c. T. P. filtrate intravenously.

Jan. 30.—10:30 a. m.: Temperature 97.6 F. Animal very ill. Leukocytes 41,400 (?). Accurate counting impossible on account of unavoidable tendency to form large clumps. 11:30 a. m.: Animal moribund. Chloroformed.

Autopsy: Heart normal. Lungs hyperemic in lower lobes. No enlarged lymph glands. Spleen of normal size and appearance. Peyer's patches not perceptibly enlarged. The liver shows several pin-point-sized whitish spots on cut surface. These are barely visible to the naked eye and are not surrounded by a zone of hyperemia. Otherwise, the liver shows nothing noteworthy. The kidneys are of about normal size. The edge of the cut surface tends to roll outward very slightly. The cut surface is moist, pale, and the cortical markings indistinct. The urinary bladder is distended with urine, which contains albumin and many granular casts.

Microscopic examination: The most striking change seen in the sections is the enormous number of hyaline thrombi in the vessels of all organs, especially in the lungs and liver. The minute whitish spots in the liver are found to be areas of necrosis infiltrated with polymorphonuclear leukocytes. These are within the lobules, perhaps a little nearer to the periphery than to the central vein. There is no vascular reaction around them. Similar areas are present in the cortex of the adrenal.

DISCUSSION AND CONCLUSIONS

From the results detailed here, it would seem that the presence of fermentable sugar in cultures of *B. typhosus* not only influences its metabolism, but may produce striking changes in the serologic reactions of the organism and may even affect the potency of its endotoxin.

It is possible that this may be a factor in the favorable results reported by Coleman¹³ from his high calory diet in typhoid fever. The large carbohydrate component of the diet could hardly be expected actually to increase the blood agar, but it would lead to a greater storage of carbohydrate in the liver and tissues. It is not impossible that the presence of this greater store of carbohydrate may render the infecting strain of typhoid bacillus more susceptible to the action of the normal and immune lysins of the blood and may also reduce the potency of its endotoxin. So far as we are aware, there are no published records of repeated blood cultures on patients given the high calory diet.

13. Arch. Int. Med., 1915, 15, p. 887.

THE LESIONS IN EXPERIMENTAL INFECTION WITH BACTERIUM TULARENSE *

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In 1911 McCoy¹ discovered a disease among the ground squirrels of California the lesions of which resembled somewhat closely, at least in the gross features, those produced in rodents by *Bacillus pestis*. In 1912, with Chapin, McCoy² reported the isolation of the microbic cause of these lesions, and to the organism he gave the name *Bacterium tularense*. This organism was exceedingly pathogenic for rodents and for monkeys, an observation which led McCoy to suggest in a letter to Wherry³ that it might be expected sooner or later to be encountered in human infections. This prophecy materialized: in 1914 Wherry and Lamb⁴ reported finding *B. tularense* in a case which they had been investigating for Dr. Vail,⁵ under the suspicion that it might be glanders.

This first patient was a meat cutter in a restaurant. At the time of examination he was suffering from an acute ulcerative conjunctivitis, enlarged cervical lymph glands, fever, and prostration. Later, a second case was sent them by Dr. Sattler,⁶ in which the lesions and symptoms were identical with those observed in the first case.

With the organism isolated from these cases, Wherry and Lamb made a considerable number of inoculations of laboratory animals, the organs of which were referred to the writer for study. In each instance practically the same lesions were found, and variations, when they occurred, were chiefly ones of size. The gross lesions have been described by Wherry and Lamb.⁴

The following are descriptions of the typical lesions as they appeared in the different organs.

* Received for publication July 19, 1915.

1. Bull. Hyg. Lab., U. S. P. H. and M.-H. S., 1911, 43.

2. Ibid., 1912, 53.

3. U. S. Pub. Health Rep., 1914, 29, p. 3387.

4. Jour. Infect. Dis., 1914, 15, p. 331. Jour. Am. Med. Assn., 1914, 63, p. 2041.

5. Ophth. Rec., 1914, 23, p. 487. Jour. Mich. Med. Soc., 1915, 14, p. 3.

6. Arch. Ophth., 1915, 44, p. 265.

Tissues were fixed in alcohol or Zenker's solution and imbedded in paraffin. They were stained by Gram's method, with hematoxylin and eosin, eosin and methylene blue, carbolthionin, and Borrel's stain.

In the skin, the lesions were somewhat diffusely necrotic in type, and in them, altho there were numerous polymorphonuclear leukocytes, the appearances indicated that tissue necrosis was the primary lesion and that this was followed by invasion of leukocytes. The picture was that of a not sharply circumscribed, rapid, necrotic process which involved all layers of the skin and subcutaneous tissue. The subjacent muscle was affected to a less extent. There were evidences in the necrotic areas of focalization of the process in the immediate neighborhood of blood vessels. In these foci, there were fewer leukocytes, often none, and only the detritus of destroyed cells with scattered masses of nuclear fragments. This is the kind of lesion encountered in the abdominal wall after puncture or scarification, and is of the same type which follows infection of the conjunctiva.

The lymph glands were swollen very evidently and were the seats of exquisite sinus catarrhus and edema. Besides this generalized lymphadenoid process there were foci of focal necrosis, which were sometimes large but usually small, and located for the most part in the germinal centers. In some instances the necrosis was more extensive; in such the acute catarrhal condition was less vivid and the necrosis involved more or less the whole gland as well as the surrounding tissues. Here again, altho there was some neighboring polynucleosis, necrosis with extreme karyorrhexis dominated the picture.

The spleens exhibited practically the same lesions as the lymph glands, i. e., very numerous areas of focal necrosis, some very minute, others relatively large, many confluent. In the very small ones there was evidence of nothing but the death and rhexis of a few cells, about which there was no apparent cellular reaction, unless occasionally the surrounding cells appeared somewhat paler than those more distant from the lesions. In the larger lesions there was frequently a reaction evident about the necrotic areas, which was characterized by only moderate numbers of polymorphonuclear leukocytes. The follicles, when they were not involved in the necrosis, were generally hyperplastic, and the pulp was exceedingly congested. Occasionally whole follicles were necrotic. In no place were giant cells observed.

The liver sections, as a rule, showed more, but also, as a rule, smaller, lesions than the spleen. Here and there throughout the tissue one could see very small palely staining areas composed of but few cells which often showed nothing but the lack of stain. Also scattered about were larger areas in which the cells stained only a pale pink with little or no evidence of nuclei. In other foci the nuclei were undergoing fragmentation; and in still other and larger ones the fragmentation was extreme, the cytoplasm was less stainable, and there was a slight polymorphonuclear reaction about the areas. Removal of the débris produced in the lesions is apparently accomplished by means of polymorphonuclear phagocytes. There was no evidence of autolysis and none of participation by endothelial leukocytes.

The hepatic lesions showed no definite predilection for a particular part of the lobule, but occurred more or less indiscriminately scattered. Very small areas of capillary hemorrhage suggested, what would be supposed, that the organism reaches the organ by way of the blood stream; and yet the lesions did not predominate in the central or capillary zone of the lobules. Sometimes they appeared directly in or upon the wall of a fairly large-sized vessel, sometimes apparently in connection with the interlobular bile ducts. In one

small lesion a stellate figure, such as has been described by Wolbach,⁷ appeared. In no section could any organism be distinctly stained by any method used.

In sections from the lungs, there were areas of lobular pneumonia and diffuse inflammatory edema. The pneumonic process was, as a rule, an acute hemorrhagic one, and the alveoli were filled with erythrocytes, desquamated alveolar epithelium, and lymphocytes. There were occasional polymorphonuclear leukocytes, but these were scant as compared with the numbers encountered in the ordinary pneumonias. In the large blood vessels, however, there were increased numbers of polymorphonuclear leukocytes; in the tissues they appeared in number only about the areas of necrosis. The earliest lesions indicated that the areas of consolidation and necrosis had arisen in much the same way as those in the other organs; namely, as areas of focal death resulting from the toxic agent brought by the blood stream. Probably the bacterium had been fixed by the endothelium of a capillary, and had then reproduced, caused a necrosis of the tissue, and produced an inflammatory edema involving the neighboring alveoli, and more necrosis. At a safe distance about the lesions, the polymorphonuclears had attempted to form a protective zone. The first impression which one had from certain sections was that one was dealing with an acute caseous pneumonia. No acid-fast or other organism could be found, however.

In sections from the intestinal tract, there were no striking lesions; nothing, for instance, which approximated those seen in the regional lymph glands draining the point of superficial infection. The lymphoid follicles were moderately enlarged. They showed no evidence of necrosis and no increased number of polymorphonuclears. Upon the mucous membrane, which was moderately congested, there was a slight catarrhal exudate and nothing more.

The kidney sections showed extreme edema and cloudy swelling, almost an acute necrosis. There were no areas of necrosis and no areas of cellular infiltration.

There were no obvious changes in the central nervous system, in the heart, or in the adrenals, tho the latter organs were congested.

According to Ledingham,⁸ the lesions of spontaneous rat plague in the spleen are necrotic in type, with or without polymorphonuclear leukocytes. Associated with these lesions, there are pulp hemorrhages. The liver lesions are karyorrhectic. In the lymph glands there are no capsule changes. There are karyorrhexis of lymphoid cells and a very large amount of nuclear detritus lying free or included in large endothelioid phagocytic cells. There are many large cells of endothelioid type throughout the nodes and many of these show mitotic figures. The lesions, therefore, are essentially focal necroses to start with, which may become abscesses, or which may show merely karyorrhexis with endothelial stimulation and proliferation. In tularensis infections, the lesions are essentially areas of necrosis with, in no case, suppuration, except in the case of the skin. There is no evidence in any section of any marked proliferation on the part of the endothe-

8. Jour. Hyg., 1907, 3, p. 359.

7. Jour. Med. Research, 1911, 24, p. 243.

lial cells, as is true in plague and typhoid. In the smallest lesion in the liver, where the process is limited and clearest, the earliest change seems to be degeneration of the liver and endothelial cells with very early karyorrhexis. In this stage there are a few lymphocytes present but no polymorphonuclear leukocytes. The latter move to the region of the lesions following the degeneration of the cells and never occur within the lesions in large numbers as in plague. Were there more endothelial proliferation, the lesions might resemble those of typhoid.

THE VARIETIES OF PNEUMOCOCCI CAUSING LOBAR PNEUMONIA, WITH ESPECIAL REFERENCE TO THEIR BIOLOGIC DIFFERENCES *

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REACTIONS OF AGGLUTINATION

Experimental evidence now at hand indicates that during the course of lobar pneumonia there is a definite biologic reaction characterized by the formation of antipneumococcic substances in the body of the patient. These antibodies are of primary importance in bringing about a favorable termination of the disease. Attempts to utilize this fact in the treatment of pneumococcal infections have led to the important observation that pneumococci vary widely in their serologic reactions.

Eyre and Washbourn¹ found that a given antipneumococcic serum would protect rabbits only against certain strains of pneumococci. Bezançon and Griffon² confirmed this observation by experiments in both agglutination and protection. The researches of Neufeld and Händel³ indicate that pneumococci might be classified into different groups according to their biologic differences, and the practical application of this principle was made by Dochez and Gillespie⁴ in America and Lister⁵ in South Africa.

Dochez and Gillespie found that pneumococci obtained from cases of pneumonia could be classified into 4 groups according to their biologic and cultural differences. A highly immune serum was prepared for each of 2 groups (I and II) and all the organisms not affected by these sera, except pneumococcus types (Group III), were placed in an atypical group (IV). These observers found that the most common type of organism in New York City during the season of 1912-13 was that of Group I. In 1913-14 the percentage incidence of the typical groups (I and II) did not change, but Group II became the dominant type.⁶

A similar study has been made of the cases of lobar pneumonia entering the Cook County Hospital during the season of 1914-15, the results of which are recorded in this paper. I am greatly indebted to the members of the medical staff of the Cook County Hospital for the clinical material so willingly placed at my disposal.

* Received for publication July 26, 1915. This work was done under a grant from the Fenger Memorial Fund.

1. British Med. Jour., 1899, 2, p. 1247.

2. Ann. de l'Inst. Pasteur, 1900, 14, p. 449.

3. Arb. a. d. k. Gsndhsamte., 1910, 34, p. 293.

4. Jour. Am. Med. Assn., 1913, 61, p. 727.

5. Specific Serologic Reactions with Pneumococci from Different Sources, 1913.

6. Jour. Exper. Med., 1915, 21, p. 114.

TECHNIC

Blood cultures were made in the usual manner. A mixture of 3.5 c.c. of blood with 100 c.c. of plain broth (0.7% acidity to phenolphthalein) was incubated for from 1 to 6 days at 37 C. Sputum cultures were made according to a method advised by Cole and Dochez.⁷ A small amount of sputum was obtained by having the patient cough and expectorate into a wide-mouthed bottle or Petri dish. A small particle of this sputum, thoroughly washed, was ground up in a sterile mortar with 12 c.c. of normal salt solution or plain broth and this mixture injected into the peritoneal cavity of a mouse. The mouse was killed in from 6 to 24 hours and cultures made from the heart's blood and the peritoneal exudate. Whenever enough time had elapsed after inoculation (18-24 hr.), a pure culture of pneumococci was usually obtained from the blood, as well as from the peritoneal exudate. Smears of the ground sputum on blood agar plates usually gave a luxuriant growth of pneumococci. Since only typical cases of lobar pneumonia were studied, the identification of the organism in subcultures was not difficult. All grades of variation, however, were encountered in the different cultural reactions.

Bacteriologic examination was made in 142 cases of acute lobar pneumonia during the active stage of the infection. In 128 of the cases blood cultures were made, in 97 or 75% of which the pneumococcus was isolated in pure culture. In 31 instances the blood cultures were negative, but in many of these the blood was examined late in the course of the infection. Usually the blood cultures were positive in from 24 to 48 hours; in a few it required from 4 to 6 days for an appreciable growth to take place. This was especially noticeable in the cultures obtained after the fifth day of the disease, and was probably due to the marked decrease at this time in the number of organisms in the blood. In 6 cases both the blood and sputum were examined, similar organisms being obtained from both sources. Of the remaining 14 cases, the sputum alone was examined. The organisms were typical pneumococci, in most instances, and highly virulent, as evidenced by the rapidity with which the mouse became ill after inoculation. The organisms often invaded the blood within 18 hours. When plate cultures were made, 2 types of pneumococci were sometimes isolated, which, in a few instances, could be differentiated by their cultural differences on blood agar plates. This discrepancy between the plates and the mouse cultures may be explained by assuming that the avirulent organisms are overgrown in the peritoneal cavity of the mouse by a more virulent strain. The characteristic inulin reaction was present in almost all instances in which the organism was studied early after isolation; this acid-forming power decreased however with the continued growth of

7. Personal communication.

the organism on artificial media. A few of the strains of pneumococci, with continued growth on blood agar, have become slightly hemolytic.

AGGLUTINATION TESTS

The technic used in these tests was similar to that used by Cole (to whom I am indebted for specific sera of Groups I and II with marked agglutinative powers) and his associates. Equal parts of a suspension of the pneumococci in normal salt solution and the sera of Groups I and II were mixed in small test tubes. The mixtures were incubated at 37 C. for 2 hours and if agglutination was not complete at the end of this time they were placed in the refrigerator for 24 hours before a final result was recorded. Agglutination often occurred within the first hour; a negative reaction, however, could only be determined after a period of 24 hours. Each strain of pneumococcus was studied by these methods as soon after isolation as possible. When there was partial agglutination with both sera, varying dilutions of each serum were used in the tests. The results of the tests are recorded in Table 1.

TABLE 1
BIOLOGIC CLASSIFICATION OF THE DIFFERENT STRAINS OF PNEUMOCOCCI AS DETERMINED BY
THEIR REACTIONS OF AGGLUTINATION

Group	No. of Cases	Percentage of Total
I.....	50	45.0
II.....	25	22.5
III.....	5	4.5
IV.....	31	28.0

Of the 111 strains of pneumococci studied, 50 were found to belong to Group I, 25 to Group II, and 5 strains were typical members of the pneumococcus mucosus group. The remaining 31 strains, atypical in their biologic reactions, were considered as members of Group IV, or the heterologous group.

The members of Group I comprised 45% of the total number of cases. With the organisms of Group I the agglutination was very striking, taking place usually in a very short time. In 22.5% of the cases an organism belonging to Group II was isolated. With these organisms the agglutination was less striking than with the members of Group I, and often little change could be detected in the mixtures after 2 hours' incubation. In most instances it required 24 hours for the reaction to take place.

There were 31 atypical strains of pneumococci that could not be classified biologically or culturally. Many of these organisms were partially agglutinated by both Sera I and II. In some instances the action was more pronounced with one than with the other. When the

organism was partially agglutinated by both sera, different dilutions of the sera were used. Usually little was gained by this procedure, for agglutination disappeared in very low dilutions. In two instances (Strains 134 and 143), however, the organisms were partially agglutinated by both sera dilutions as high as 1:128. It is interesting to note at this point that the typical members of Groups I and II are usually not agglutinated by their homologous sera in high dilutions. Fifteen of the 31 organisms of the heterologous group were not agglutinated by either of the specific sera.

In Types I, II, and IV the morphology was practically the same. Variations in size, shape, and arrangement of the organisms were as frequent in one of these groups as the other. Culturally, however, the members of Group II gave usually a more moist growth than did those of Group I, but the capsules were not appreciably larger. Organisms of the mucosus type produced abundant mucus on blood agar plates; they were uniformly small, surrounded by a heavy capsule. The biologic characteristics of the strains of pneumococci grown on artificial media since their isolation have not varied.

MORTALITY

The cultures were made on early cases of lobar pneumonia without regard to age or any other factor which influences mortality—a few of the cases being moribund at the time cultures were taken. In

TABLE 2
THE RELATION OF THE BIOLOGIC DIFFERENCES OF PNEUMOCOCCI TO MORTALITY

Group	Recovered	Dead	Mortality
I	37	13	26.0%
II	18	7	28.0%
III	1	4	80.0%
IV	23	8	25.8%
Negative cultures	25	6	19.3%
Total	104	38	26.7%

Table 2 the mortality for each group is recorded. Thirty-eight of the 142 cases resulted fatally,—a mortality of 26.7%. The lowest mortality was in Groups I and IV. In Group II the mortality was somewhat higher, and in Group III, 80% of the cases terminated fatally. In the cases in which negative blood cultures were obtained, the death rate was small (19.3%); this supports the view that in favorable cases the organisms disappear from the blood early in the course of the infection.

The relation of age to the incidence and mortality of lobar pneumonia is shown in Table 3. Young, healthy adults between the ages of 20 and 40 years are most often infected. The mortality is strikingly low during the first three decades, but increases greatly with each succeeding decade.

The severity of the course and the clinical picture varied in all the groups, so that there were no clinical observations that could be considered peculiar to one single group. Usually, however, in the Group I series the clinical picture was relatively typical, and a crisis was more common than lysis when there was a favorable termination. Complications were not uncommon, and a few individuals suffered two attacks of pneumonia at short intervals. In one of these cases a careful bacteriologic study was made, the findings of which were of sufficient interest to report.

TABLE 3
THE RELATION OF AGE TO THE MORTALITY IN LOBAR PNEUMONIA

Decade	Recovered	Dead	Mortality
10-20	5	0	0
20-30	34	2	5%
30-40	36	15	29%
40-50	16	12	42%
50-60	9	6	40%
60-70	4	3	43%
Total	104	38	26.7%

TWO ATTACKS OF LOBAR PNEUMONIA IN THE SAME INDIVIDUAL, CAUSED BY PNEUMOCOCCI WITH DIFFERENT IMMUNOLOGIC REACTIONS

A second attack of lobar pneumonia in the same individual is not an uncommon occurrence in hospital practice. While the symptoms of the first attack are abating and recovery seems assured, the patient suddenly develops a chill and another typical pneumonia ensues. In the absence of contradictory evidence the natural supposition has been that both infections are caused by the same organism. Other possibilities, however, must be considered. Is such an occurrence a new infection by a different organism, or a variation of the organism causing the first infection? Or may it be a latent process activated by the debilitating effects of the first attack? The following incidence of such an infection is important in this connection:

CASE 68.—A laborer 56 years of age, in the medical service of Dr. Capps and Dr. Post, entered the Cook County Hospital, Feb. 6, 1915, complaining of a severe cough, high fever, and pain in the right side of the chest. His sickness had begun the day before with headache and a cough. These symptoms

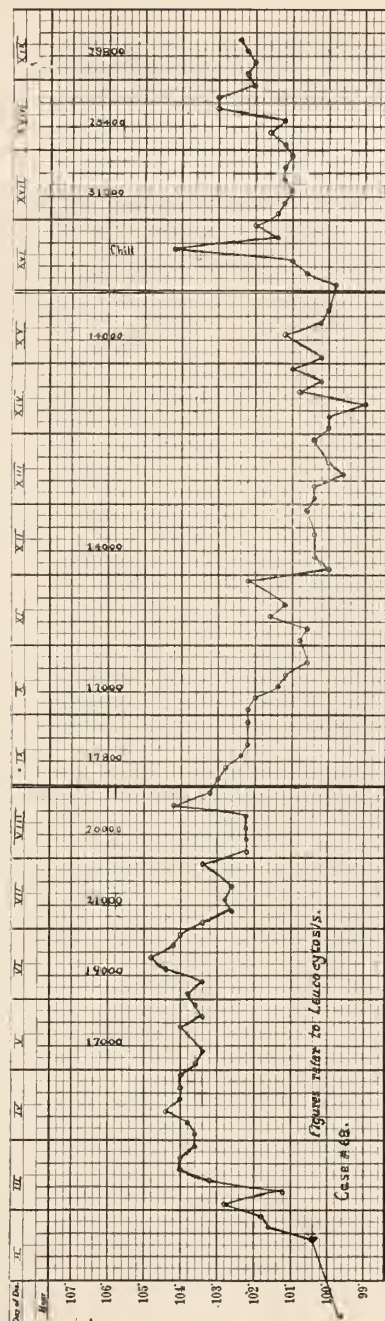


Chart 1.—The temperature and leukocytes in the case of two attacks of pneumonia in the same individual.

had rapidly increased and he had had a severe chill a few hours before his entrance into the hospital. Following the chill all the subjective signs of a lobar pneumonia appeared. With the exception of a chancre 20 years before, the remainder of the history is unimportant.

He was well nourished. His respirations were rapid, shallow, and painful. The throat and tonsils were reddened. On the right side of the chest, at the level of the fourth interspace anteriorly, and at the angle of the scapula posteriorly, there were areas of dullness, bronchial breathing, and bronchophony. Over the remainder of the right chest, the breath sounds were distant; numerous moist râles were to be heard throughout respiration. The left lung was unchanged. The left border of the heart was 11 cm. to the left of the midsternal line; the heart sounds were regular and clear, without audible murmurs. At the time of this examination the temperature was 104 F., pulse 98, and respiration 48. The sputum was thin, blood-tinged, and stained smears revealed numerous gram-positive, encapsulated diplococci. There was a polymorphonuclear leukocytosis of 17,000 per c.mm. The urine was normal, except for a decreased chlorid content. The systolic blood pressure was 85 mm. and the diastolic 65 mm. of mercury.

All the signs and symptoms of an acute lobar pneumonia involving the right middle and lower lobes of the lung intervened (see Chart 1); the course was severe and the patient, delirious. On the sixth day an acute, purulent arthritis of the left shoulder developed. The joint was drained 3 days later, a thick, yellow, purulent material being obtained from which a pneumococcus was isolated in pure culture. The temperature began to fall by lysis on the seventh day. The symptoms rapidly abated and by the twelfth day resolution was in progress and the temperature was 99 F. per rectum. The patient felt fairly well until the fourteenth day, when he suddenly had a violent chill while all the symptoms and signs of a lobar pneumonia involving the lower left lobe of the lung intervened. Respirations increased from 24 to 56; the leukocytes from 14,000 to 31,000 per c.mm. The symptoms rapidly increased and the patient died Feb. 23, 1915.

Anatomic Diagnosis (Dr. Herzog).—Lobar pneumonia of both sides, with red and gray hepatization; fatty and parenchymatous degeneration of the myocardium; marked dilatation of the right side of the heart; parenchymatous degeneration and fatty changes of the liver; passive hyperemia of the kidneys; metastatic purulent arthritis of the left shoulder.

Bacteriologic Examination.—Typical pneumococci were isolated from the blood in 3 different instances,—on the third and fourth days of the first attack, and on the third day of the second attack. Pneumococci were isolated also from the purulent material from the infected shoulder joint and the sputum. The organisms were all similar morphologically and culturally but different in their reactions of agglutination. The blood cultures made during the primary infection, and the culture made from the joint, yielded an organism which was agglutinated by the serum of Group I. The organism isolated from the blood and sputum during the second attack was not agglutinated by either the serum of Group I or that of Group II. Morphologically, however, the organisms were similar. On blood agar plates the colonies were green-colored and slightly more moist than those of the first infection. Both organisms were encapsulated, and in their cultural reactions typical of the pneumococcus group.

In this case there were two different pneumococcus infections caused by organisms with different biologic reactions. The first infec-

tion was caused by an organism with the biologic characteristics of Group I; the terminal infection was caused by a member of the heterologous group. Was this new organism derived from the external world, or was it a variation of the pneumococcus already present in the body in large numbers? The latter explanation seems the more probable, for in the body of an individual suffering from lobar pneumonia the environmental conditions are ideal for a change in the biologic characteristics of the invading pneumococcus. Rosenow⁸ has shown that these biologic reactions may change under experimental conditions. By varying the media on which pneumococci are grown, cross agglutination between Groups I and II can be obtained. Hence, if these observations are correct, it seems reasonable to assume that the same organism may be the causative factor in both infections, the immunity perhaps being overcome, as suggested, by a change in the biologic reaction of the pneumococcus.

SUMMARY

The pneumococci isolated from the blood and the sputum of patients suffering from lobar pneumonia vary widely in their biologic reactions. By means of agglutination tests with specific immune sera, these pneumococci may be classified into certain biologic groups. There are many atypical organisms, however, exhibiting the biologic characters of both groups to a limited extent. With the exception of the pneumococcus mucosus type, these different groups have no distinguishing morphologic or cultural peculiarities. Group I is the most typical group, but its members are less virulent than those of Groups II and III. Group IV is the least virulent of the four types.

During the winter of 1914-15 the percentage incidence of the typical groups (I, II, and III) in the vicinity of Chicago was 72%, and 28% for the heterologous types (Group IV). These figures are approximately the same as those obtained by Dochez and Avery in New York city during the two preceding seasons. Group I was the dominant type, and Group IV was more frequent than Group II or III. This predominance of the less virulent groups (I and IV) is again indicated by the very low mortality (26.7%) for the series.

Repeated attacks of pneumonia occurring at short intervals in the same individual may be caused by pneumococci with different biologic reactions.

8. Jour. Infect. Dis., 1914, 14, p. 1.

THE IMPORTATION OF TYPHUS FEVER INTO THE UNITED STATES *

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It is not unreasonable to suppose that the present European conflict will give rise to a typhus visitation of this country similar to that which followed the recent Balkan war.¹ Altho the writer does not believe that typhus fever can make any appreciable headway amongst a people who are in general free from vermin infestation, it is well to know exactly what we might have to face; hence, a little closer study of the data available in this connection does not seem untimely.

Typhus fever being endemic and epidemic in Europe and Africa, Asia, and Mexico, this country is theoretically exposed on three sides to primary sources of infection. Importation across the Pacific is not very imminent so long as the exclusion of oriental labor remains a national policy, and so far as the writer knows, no cases are on record as having been introduced from this direction. Importation from Mexico, which was feared by Wilder,² has actually come to pass: the recent Mexican rebellions have been responsible for several scattered cases in the Southwest, and for a small epidemic among our New Mexico Indians.³ Importation across the Atlantic, however, possesses the greatest epidemiologic interest. While it is theoretically true that "the period of incubation of typhus fever is of sufficient duration to allow an immigrant to take passage after infection and reach a United States port without showing evidence of the disease,"⁴ it must nevertheless be borne in mind for practical purposes that no cases have been imported on ocean "greyhounds" and that the average typhus-bearing ship crosses the Atlantic in about 2 weeks. When it is further considered that the incubation period in man is usually estimated at from 7 to 14 days, and probably does not exceed 21 days (Wilder), it must be admitted that the great majority of persons

* Received for publication July 27, 1915.

1. U. S. Pub. Health Rep., 1914, 29, p. 677. Ann. Rep. Health Officer Port of New York, 1914, 5, p. 66.

2. Jour. Infect. Dis., 1911, 9, p. 97.

3. U. S. Pub. Health Rep., 1911, 26, p. 1112; 1914, 29, p. 1068. Day: New Mex. Med. Jour., 1914, 12, p. 45.

4. U. S. Pub. Health Rep., 1914, 29, p. 677.

directly infected in Europe will have developed the disease sufficiently during transit to be readily recognized, on their arrival in this country, as cases of typhus fever. Moreover, if we adhere to the practice of quarantining the steerage passengers of such typhus ships until the maximal incubation period is over (as was done by O'Connell in New York), the danger of admitting cases of typhus infected in Europe will be reduced to a minimum.

However, there seems to be increasing evidence that not all cases of typhus imported into this country were infected before taking passage, and that the quarantining of incoming patients and of their contacts will not prevent the continued introduction of certain sporadic cases and their dissemination throughout the country. In fact, the question of the rôle played by immune carriers of typhus lice and the mechanism of typhus importation by such individuals does not seem to have attracted much attention in discussions of the subject. The main features in this connection may be well brought out by study of an illustrative case, such as the writer had the opportunity of making in the person of a Macedonian immigrant.⁵ The man arrived at East Syracuse March 24, 1915, remained well until April 7, when he began to complain of mild prodromal symptoms, and came down acutely on April 9. From then on, until defervescence on April 19, he ran the typical course of typhus fever, terminating in recovery. The clinical diagnosis was confirmed by a strongly positive reaction of agglutination. (All the immunity tests referred to in this study were made by Dr. Harry Plotz and Dr. Peter K. Olitsky, of the Mount Sinai Hospital, New York City.)

An inquiry into the recent history of this patient brought out some interesting features. He had made three previous visits to America, working as a common laborer in different cities, and returning each time to his home near Florina, Province of Monastir, European Turkey. According to his statement, there was no typhus fever at or near Florina during his last visit. His return to this country may be presented in tabular form as follows:

Left Florina by rail.....	March 1, a. m.
Arr. Salonika	March 1, p. m.
Left Salonika by packet	March 2

5. The clinical features of this case are discussed by Levy and Kantor: *Boston Med. and Surg.*, 1915, 73, p. 473.

Arr. Piraeus	March 3
Left Piraeus by S. S. "Patris"....	March 3
Left Kalamata	March 4
Left Patras	March 5
Left Algiers	March 10
Arr. New York	March 23, a. m.
Left New York by rail.....	March 23, p. m.
Arr. E. Syracuse	March 24

The itinerary of the "Patris" was confirmed by Dr. J. J. O'Connell, health officer of the Port of New York, who further reports that there was no typhus notified at the ports of call on the dates mentioned. At Salonika, however, there were 6 fatal cases of the disease during the two weeks following February 21.⁶

According to the patient's story, he was free from vermin until he boarded the packet at Salonika. Both that ship and the "Patris" were apparently in poor sanitary condition, for by the time the latter arrived in New York many of the steerage passengers harbored body lice. As already mentioned, the subject did not show any symptoms of the disease until April 7. Since he took pains to destroy his infected clothing immediately upon his arrival at East Syracuse, the basis for contagion was eliminated, and no secondary cases developed.

Two strikingly similar cases⁷ occurred in Massachusetts. The immigrants arrived at Boston on April 23, 1914. One of them proceeded to Graniteville, where he was taken ill with typhus on May 8. The other went to Quincy, where he came down with typhus on May 3. The S. S. "Rhaetia," on which the subjects arrived, left Hamburg on April 8 and Boulogne-sur-Mer on April 9. No sickness developed on board during the passage, and no cases of typhus were reported at either of the ports of departure (according to information submitted by Dr. F. X. Crawford, port physician, city of Boston).

A study of these cases seems to justify the following generalizations: (1) All three were isolated cases. According to available information, typhus did not exist at the ports of departure. No cases

6. U. S. Pub. Health Rep., 1915, 30, p. 1564.

7. Ibid., 1914, 29, p. 1381.

developed on shipboard. Therefore, no ordinary means of quarantine could have prevented their importation. (2) In each instance, the space of time elapsing between the date of embarkation and the development of the disease in this country exceeded the maximal incubation period; namely, 34 days in the Syracuse case, 29 days in the Graniteville case, 24 days in the Quincy case. (3) In each instance, the space of time elapsing between arrival in this country and the development of the disease fell within the incubation limits of typhus fever; namely, 2 weeks in the Syracuse and Graniteville cases, 10 days in the Quincy case. (4.) It follows, therefore, that the infections must have taken place on shipboard. However, typhus is not a ship disease any more than it is a house disease. Besides, vessels sailing from certain suspected ports are now being fumigated before their departure (as was the case with the "Patris") for the destruction of rats and other vermin. On the other hand, the acceptance as passengers of typhus carriers (i. e., immune carriers of typhus lice) is not prevented, and the conditions on shipboard in the steerage, during each voyage at least, still appear to be eminently favorable for the spread of contagion. It seems much more likely, therefore, that the infections in these cases came from lice harbored by immune fellow-passengers rather than from vermin previously existing in the vessels of passage.

Let us consider a little more closely the question of typhus carriers. It is obvious that under appropriate circumstances any typhus immune may become a typhus carrier. In order to obtain some idea as to the actual existence of such persons, tests were performed on four of the Syracuse patient's associates, all Macedonians. Altho these men were in good health and denied ever having been sick with typhus, two of them gave distinctly positive agglutination and complement-fixation. Furthermore, in their studies on typhus immunity, Plotz and his co-workers found that uninfected individuals may have a certain degree of (natural?) resistance to the disease in question. Finally, all convalescents from the endemic Brill's disease are typhus immunes. It thus appears that we have in this country a by no means negligible group of individuals who may act (or perhaps who have acted) as typhus carriers or distributors under favorable circumstances.

Of great interest in this connection is the question of American endemic typhus, or Brill's disease. A survey of the reported cases

indicates that the malady has been recognized in most of our large cities from Massachusetts to Virginia and from New York to Minnesota and that probably several hundred new cases develop annually. (Cases of Brill's disease have been reported to date from the following states: New York, Massachusetts, Pennsylvania, Maryland, Virginia, Georgia, Indiana, Illinois, Wisconsin, Minnesota, as well as from Washington, D. C.) What is the origin of this disease? What is its exact relation to the epidemic fever? Why is it so much milder in its manifestations? It seems to the writer that we are now in possession of sufficient data to offer a tentative hypothesis for the explanation of these phenomena. Perhaps some such theory as the following may stimulate further investigation and thus aid in definitely solving the problem in question:

Individuals actually sick with European typhus have never entered this country in large numbers. Only exceptionally have isolated cases been imported, and whatever small epidemics have resulted have been checked more or less promptly. On the other hand, the importation of typhus lice by immunes must have been going on ever since the establishment of communication between this country and the typhus infested districts of Europe. However, being imported by immune individuals (according to our theory), the typhus germ would be deprived of the opportunity to sustain its virulence by passage through a human host. Indeed, it might be conceived to undergo a certain degree of attenuation during its sojourn of about 2 weeks in the body of the originally infected louse or its descendants. If, now, large numbers of such attenuated germs were actually introduced into the United States in the manner suggested, might they not be expected to give rise, in susceptible individuals, to a form of mild typhus corresponding in its manifestations to Brill's disease as we know it to-day? In other words, may we not look upon American typhus simply as European typhus modified by an unusually long passage—as compared with epidemic conditions—through a non-human host?

SUMMARY

The isolation of immigrants sick with typhus fever and of their contacts, does not prevent the introduction of the disease into this country.

It is very probable that immune carriers of typhus lice, by infecting individuals on shipboard, have caused the importation of cases of epidemic typhus fever.

It is possible that Brill's disease may be the result of the introduction into this country (by immune carriers) of lice harboring attenuated typhus bacilli.

In order to prevent the further importation of all forms of typhus fever, attention should be directed not so much against individuals, as against lice,—the actual carriers and transmitters of the disease in question.

NOMENCLATURE OF THE COCCACEAE *

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The naming of bacterial species, genera, and higher groups, indeed the whole subject of bacterial nomenclature, is in a condition which can best be described as chaotic. Little, if any, advance has been made in the last two decades. The chief reasons for this state of affairs, pointed out at different times by various authors, may be summarized in the statement that many bacteriologists have ignored the rules or laws which are generally recognized by biologists to govern nomenclature. Even in those cases where there is an expressed purpose to be guided by fixed rules, the rules have not been well defined, and in some instances have not been compatible with best usage.

It is clearly recognized today that there exist among the so-called bacteria, forms which intergrade with the protozoa. While by many writers some of these are classed with animals, most of the organisms usually included with the bacteria are regarded rather definitely by biologists as plants, or at least as being plant-like in most of their characteristics. It would seem, therefore, that in so far as it is practicable, the botanical rules of nomenclature should be followed in naming the bacteria. Some authorities, it is true, have proposed that unicellular forms in general be placed in a separate "kingdom," the Prostista. From the standpoint of nomenclature this increases the difficulty by necessitating two points of contact among the kingdoms instead of one. It may not be easy to differentiate the lower plants from the lower animals, but there is just as great difficulty in separating higher plants from lower forms.

Both botanists and zoölogists have adopted codes of nomenclature at representative international congresses. No separate code for bacteriologists has ever been adopted or proposed. It is apparent, therefore, that so far as it is adapted, the bacteriologist should follow the botanical code in his classification of the bacteria. The protozoölogists have apparently been more successful than the bacteriologists (using this term in the narrow sense) in their naming of micro-organisms, for in most cases they have conformed with a measurable

* Received for publication July 27, 1915.

degree of success to the rules of zoölogical nomenclature. Bacteriologists, on the other hand, have usually followed their own bent. The present uncertainty in names is the result. Without adherence to definite standards there would seem to be no hope for improvement. However, a careful study of the international code of botanical nomenclature will show that it can be applied practically without any essential modification to the naming of groups among the bacteria quite as well as among the higher plants.

Probably the most successful and useful study of a considerable group of bacteria made in recent years is that of the Winslows (1906 and 1908) on the family Coccaceae. They have formulated apparently as satisfactory a classification of the group as a whole, as the present state of our knowledge would seem to admit. They have definitely expressed themselves in favor of following recognized rules, as is evidenced in their chapter on "Bacterial Classification," where they state:

If the Linnaean system is to be used among the bacteria, however, it should be used correctly. Much of the confusion in bacteriological literature results from neglect of the simple rules of nomenclature. The principle that a species should bear two Latin names, generic and specific, and two names only, has been ignored by many medical workers; and few bacteriologists, except Migula and Chester, have respected the principle of priority which requires that a species shall bear the name given to it in the first published description sufficiently full for identification.

With a view to checking the names applied by the Winslows to genera and higher groups, the writer has studied them from the standpoint of priority, validity, and suitability. Many of the names used by these authors are valid, but others apparently contravene the rules to which they have subscribed.

The Winslows (1908) divide the Coccaceae into subfamilies and genera as follows:

Subfamily A. Paracoccaceae Winslow and Rogers.

Genus I. *Diplococcus* (Weichselbaum) Winslow and Rogers.

Genus II. *Ascococcus* (Cohn) Winslow and Rogers.

Genus III. *Streptococcus* (Billroth) Winslow and Rogers.

Genus IV. *Aurococcus* Winslow and Rogers.

Genus V. *Albococcus* Winslow and Rogers.

Subfamily B. Metacoccaceae Winslow and Rogers.

Genus VI. *Micrococcus* (Hallier, Cohn) Winslow and Rogers.

Genus VII. *Sarcina* (Goodsir) Winslow and Rogers.

Genus VIII. *Rhodococcus* Winslow and Rogers.

These subfamilies and genera will be discussed below solely with respect to the apparent validity of the names used for the various groups, and wholly irrespective of the characteristics used by these authors in the diagnosis of the groups.

Paracoccaceae Winslow and Rogers.—This is a subfamily created by Winslow and Rogers to include those genera of the family Coccaceae which conform to the following diagnosis: "Parasites. Growth not abundant (or, one species, zoogloea-forming saprophytes. Growth abundant in saccharose media). Generally Gram-positive. Acid formers."

Article 23 of the International Rules for Botanical Nomenclature states: "Names of subfamilies are taken from the name of one of the genera in the group, with the ending—*oidae*." The subfamily name Paracoccaceae does not conform to either of these requirements. No genus Paracoccus has ever been described. Probably the most characteristic genus belonging to this group, at least the one which is most commonly recognized, is Streptococcus. A suitable subfamily designation would therefore be Streptococcoideae. It may be recalled, however, that a subfamily is a group interpolated between the groups family and tribe when such additional grouping appears to be desirable. It may be appropriate therefore to reduce the subfamily to a tribe. The name of a tribe, according to Article 23 of the code, should be taken from the name of one of the constituent genera with the ending—*eae*. Such a tribe, Streptococceae, was created by Trevisan (1889). An emendation of the tribal description given by this author could be made to cause it to conform to the subfamily diagnosis of Paracoccaceae.

It would therefore seem that strict adherence to the rules of nomenclature would necessitate that the tribal designation Streptococceae Trevisan be substituted for the invalid subfamily designation Paracoccaceae Winslow and Rogers.

Diplococcus (Weichselbaum) Winslow and Rogers.—In the designation of this genus, as well as in many of the succeeding, the method of indicating the author or the authority for the name as used by the Winslows does not appear to be in conformity with Article 41 of the code. This reads as follows:

"An alteration of the constituent characters or of the circumscription of a group does not warrant the quotation of another author than the one who first published the name or combination of names. When the changes have

been considerable, the words: *mutatis charact.*, or *pro parte.*, or *excl. sp.*, *excl. var.*, or some other abridged indication, are added after the citation of the original author, according to the changes which have been made, and of the group in question. Example: *Phyllanthus* L. *em. (emendavit)* Müll. Arg."

It is evident, therefore, that this name should have been written "Diplococcus Weichselbaum," or, if the emendation is so important or drastic as to require special emphasis, "Diplococcus Weichselbaum em. Winslow and Rogers."

This name was first used (with the spelling *Diplococcus*) by Billroth (1874) to designate a growth form of his *Coccobacteria septica* in which the spherical cells occurred in pairs. It was not used as a genus. As a generic name, *Diplococcus* came into use without any definite characterization. Bumm (1885) used the term *diplococcus* (tho not in a generic sense) for the gonococcus and related forms. In no case was it used in a binomial combination. Flügge (1886) followed Bumm in the use of the name *Diplococcus albicans tardissimus*; but this is a trinomial and, therefore, invalid. In the same year Weichselbaum (1886) gave to the pneumococcus the name *Diplococcus pneumoniae*, a valid binomial. This seems to be the first correct and adequate designation of a species in the genus, and the pneumococcus should therefore be regarded as the type. Several other species were assigned to this genus in the next two years, but the writer has been able to find 4 only which appear to have binominal form and to be therefore valid. These are *Diplococcus roseus* Bumm, *D. subflavus* Bumm, *D. luteus* Adametz (1887), and *D. coryzae* Hajek (1888).

As the formal designation of a genus, *Diplococcus* has rarely found its way into classifications, altho *diplococcus* is in common use as a casual designation. For example, Sternberg (1892) under the heading of "Diplococcus" makes the following statement:

Association in pairs is common to all of the micrococci, inasmuch as they multiply by binary division. When such association has rather permanent character, it is customary to speak of the microorganism as a *diplococcus*, but we doubt the propriety of recognizing this mode of association as a generic character.

Winslow and Rogers (1905) have included *Diplococcus* as one of the valid genera in their classification of the Coccaceae. In their latest contribution on this subject (1908), the genus is defined as follows:

Strict parasites, not growing or growing very poorly, on artificial media. Cells normally in pairs, surrounded by a capsule. Fermentative powers high, most strains forming acid in dextrose, lactose, saccharose, and inulin. Hemolytic power generally lacking. Characteristic group serum reactions.

They assign to this genus the pneumococcus, the gonococcus, the meningococcus, and the coccus of catarrh. If the gram-positive cocci of which the pneumococcus is the type are to be grouped together into a genus, it would appear that the generic name *Diplococcus* is valid; if, however, the gram-negative cocci of which the gonococcus is the type are to be included in the same genus, *Diplococcus* becomes a synonym of an earlier genus *Neisseria* Trevisan. This latter genus was founded upon the gonococcus as the type by Trevisan in 1885. Article 46 of the botanical code states: "When two or more groups of the same nature are united, the name of the oldest is retained." Inasmuch as the genus *Diplococcus* as used by the Winslows contains also the type of the genus *Neisseria* Trevisan, and since the latter name has priority, it should replace *Diplococcus*. It may again be emphasized that *Diplococcus* apparently constitutes a valid generic designation for a genus of which the pneumococcus and not the gonococcus is the type.

Ascococcus (Cohn) Winslow and Rogers.—The designation of authorship as given by the Winslows is open to the objection previously noted. It should be written "*Ascococcus* Cohn," or "*Ascococcus* Cohn em. Winslow and Rogers."

The name *Ascococcus* was first used by Billroth (1874) for a growth form of his *Coccobacteria septica* in which the spherical cells are imbedded in gelatin or slime. It was not employed as a generic designation. Cohn (1875) published the name as a genus, with the species *A. Billrothii* Cohn. The generic description given by Cohn is:

"Cellulae achromaticae globosae densissime consociatae in familias tuberculosas globosas vel vales irregulariter lobatas, lobis in lobules minores sectis, capsula globosa vel ovali gelatinoso-cartilaginea crassissima circumdatas, in membranam mollem facili secendentem floccosam aggregatas."

The species described developed spontaneously in a culture medium of ammonium tartrate. The specific diagnosis is

"Familiae tuberculosae 20-160 μ . capsula ad 15 μ . crassae. In solutione ammonii tartarici acidi aëre lavata vel butyrico praeditam formanten observavi. March 1874. Haud scio citrum eandem an affinem speciem ill. Billroth in aqua carnis foetida detexerit."

Cienkowski (1878) described an organism responsible for a gummy or viscous fermentation of syrups in sugar factories. He regarded it as conforming to Cohn's conception of *Ascococcus*, and named it *A. mesenteroides*. A study of the same organism was made by Van Tieghem (1878). He concluded that this form is distinct from *Asco-*

coccus Cohn, and made it the type of a new genus *Leuconostoc*. He called attention to the points differentiating the two genera. In *Ascococcus* the cells are spherical, very small, and grouped in great numbers to form globular or oval families, which are more or less irregularly lobed. The cells are closely united, separated by a small amount of gelatinous material; each family is surrounded by a cartilaginous envelope. In *Leuconostoc* the cells are arranged in curved chains separated from each other by a considerable amount of gelatinous material, the gelatin on the exterior not being thicker than that between the chains. The *Ascococcus* of Cohn grew in ammonium tartrate solution; *Leuconostoc* in sugar, making the medium decidedly acid. Van Tieghem placed the genus *Leuconostoc* among the *Nematogenae* in Cohn's classification, while *Ascococcus* was grouped with the *Glaeogenae*. The use of the names *Ascococcus* and *Leuconostoc* in subsequent literature shows great variation.

Winslow and Rogers (1905) have revived *Ascococcus mesenteroides* Cienkowski as the type of their emended genus *Ascococcus* (Cohn) Winslow and Rogers. They conclude, because of the cheesy odor developed by the organism described by Cohn and the frequent confusion by Cohn of cocci and bacilli, that in all probability this author was really dealing with a rod-shaped organism. It would seem that they are in error in this matter, for the illustrations accompanying Cohn's description are quite distinctive. They also note Cienkowski's use of the term *Ascococcus* and state: "Van Tieghem (1878) a little later worked on the same form and substituted for *Ascococcus* the generic name *Leuconostoc* in order to emphasize the resemblance between the zoogloea-forming coccus and the blue-green *Nostoc*." This would seem to be scarcely a full statement of the case. Van Tieghem concluded that the organism of Cienkowski differed so markedly from Cohn's description of *Ascococcus* that a new generic designation was required. The Winslows "emend" the diagnosis of *Ascococcus* to fit their conceptions of the genus. It would seem that they were misled, perhaps, by the apparent appropriateness of the name *Ascococcus*. There is little question but that Cohn's *Ascococcus* was entirely distinct from *Leuconostoc*. The former name should probably be reserved for Cohn's species. However, if Van Tieghem was in error in believing that Cienkowski's organism deserved generic separation from *Ascococcus*, then the latter's name may be revived.

It is probable that Migula's (1900) conclusion that Cohn's *Ascococcus* is a growth form of *Micrococcus* is correct. The species *A. Billrothii* has apparently never been recognized with certainty since described. Unless it can be found, and shown to be worthy of generic recognition, *Ascococcus* should lapse into synonymy. For the Winslows' type, the generic name *Leuconostoc* Van Tieghem should be substituted.

Streptococcus (*Billroth*) *Winslow and Rogers*.—This name (in the form *Streptococcus*) was introduced by Billroth (1874) as a designation of a growth form of his pleomorphic species *Coccobacteria septica*. His use of the term was not generic, and he should not be quoted as the author, as has often happened (Migula, Winslow, Vuillemin, etc.). Cohn (1875) did not early recognize *Streptococcus* as a genus. He states: "Was Billroth *Streptococcus* nennt, hatte ich selbst als *Torula* form von *Micrococcus* bezeichnet." Later, however, he included the genus in the tribe Nematogenae, tho there seems to have been no species assigned to it. This is generally regarded as essential to the validity of a genus, hence Cohn is not to be quoted as the author. Ogston (1883) again used streptococcus as the designation of a form-group, not as a genus. Fehleisen (1883) described organisms now regarded as members of the genus *Streptococcus*, but without using this term.

Apparently the first valid use of the name in a generic sense was that of Rosenbach (1884). He named two species, *Streptococcus pyogenes* and *S. erysipelatos*. The genus should therefore be ascribed to Rosenbach.

Probably the genus *Streptococcus* has been more generally accepted by bacteriologists than any other genus of bacteria, with the possible exception of *Micrococcus*. In 1905 Winslow and Rogers emended the characterization of the genus. In its later form (1908) it reads:

Parasites. Cells normally in short or long chains (under unfavorable cultural conditions, sometimes in pairs and small groups, never in large packets). Generally stain by Gram. On agar streak, effused translucent growth, often with isolated colonies. In stab culture, little surface growth. Sugars fermented with formation of large amount of acid. Generally fail to liquefy gelatin or reduce nitrates.

The generic name *Streptococcus* would appear to be valid, but should be credited to Rosenbach and not to (Billroth) Winslow and Rogers.

Aurococcus Winslow and Rogers.—This generic name was first proposed by Winslow and Rogers (1906) to include the orange cocci. In its later form (1908) the diagnosis of the genus reads:

“Parasites. Cells in groups and short chains, very rarely in packets. Generally stain by Gram. On agar streak good growth, of orange color. Sugars fermented with formation of moderate amount of acid. Gelatin often liquefied very actively. May or may not reduce nitrates.”

These authors include three species, *Aurococcus aureus* (Rosenbach) Winslow, *Aur. aurantiacus* (Schröter, Cohn) Winslow and Rogers, and *Aur. mollis* (Dyar) Winslow. This genus, together with *Albococcus*, was created by splitting up the older genus *Staphylococcus* into two genera. For the generic name *Aurococcus* to be established as valid, it must be shown that the name which it displaces is invalid. It is true that in the form “*staphylococcus*” the name is used as a casual designation of a coccus grouping, but this does not invalidate *Staphylococcus* as a genus name any more than the common use of “*aster*” and “*lily*” invalidates the genera *Aster* and *Lilium* of the botanists.

Staphylococcus was first proposed by Ogston (1881 and 1883), but first used in a strict generic sense by Rosenbach (1884), who described a *Staphylococcus pyogenes aureus* and a *Staphylococcus pyogenes albus*. On a later page in the same paper, Rosenbach designates these organisms as *Staphylococcus aureus* and *Staphylococcus albus*, respectively. This genus is split by the Winslows into the two genera, *Aurococcus* and *Albococcus*, the two species of Rosenbach constituting the types. These authors thereupon discard the original name, *Staphylococcus*. Article 45 of the botanical code reads:

When a genus is divided into two or more genera, the name must be kept and given to one of the principal divisions. If the genus contains a section or some other division which, judging by its name or its species, is the type or the origin of the group, the name is reserved for that part of it.

It would seem that the Winslows have no adequate nomenclatural reasons for abandoning the generic name *Staphylococcus*. It should therefore be retained for one of their genera. Inasmuch as the *Staphylococcus aureus* was described first, and is in a sense the type species, the name *Aurococcus* should be abandoned as invalid and should be reduced to a synonym of *Staphylococcus* Rosenbach.

Albococcus Winslow and Rogers.—If the white staphylococci are to be regarded as deserving of generic recognition, the name *Albococ-*

cus would appear to be valid. It is probable that strict conformity to the rules of nomenclature would require the use of the name *Albococcus albus* (Rosenbach) rather than *Albococcus pyogenes* (Rosenbach) Winslow for the type species.

Metacoccaceae Winslow and Rogers.—A subfamily proposed by Winslow and Rogers (1905) to include genera of cocci conforming to the following description:

Facultative parasites or saprophytes. Thrive best under aerobic conditions. Grow well on artificial media, producing abundant surface growths. Planes of fission often at right angles; cell aggregates in groups, packets, or zoöglæa masses. Generally decolorize by Gram. Pigment yellow or red.

The objections to the use of a subfamily name of this form made with reference to *Paracoccaceae* hold for this subfamily. The most important of the genera included is *Micrococcus*. The subfamily might well be designated *Micrococcoideae*, or a tribe created for these genera, with the name *Micrococceae*. The latter has already been used by Trevisan (1889) as a tribal name to include several genera, of which *Micrococcus* is one. It would seem, therefore, that by an emendation this term could be used appropriately, replacing *Metacoccaceae Winslow and Rogers* by *Micrococceae Trevisan*.

Micrococcus (Hallier, Cohn) Winslow and Rogers.—The name *Micrococcus* was first used by Hallier (1866) to designate a growth form of a mold, in accordance with his theory of pleomorphism. He did not use the name in a generic sense, and the genus should therefore not be ascribed to him, as has frequently occurred with various writers. Cohn (1872) adopted the name and defined it as a genus containing very small spherical or oval organisms, with colorless or faintly colored cells, without motility, variously united into cell groups. The organism to which the name was first given was *Micrococcus prodigiosus* (Chr.) Cohn. This organism we know now to be a rod; it has therefore been removed from this genus. The next organism described was *M. luteus* (Schroeter) Cohn. This form may well be considered the type of the genus, as it has been adequately described.

The genus *Micrococcus* has been very generally recognized by bacteriologists. Winslow and Rogers (1905) emended the diagnosis of the genus. In its later form it reads:

Facultative parasites or saprophytes. Cells in plates or irregular masses (never in long chains or packets). Generally decolorize by Gram. Growth on agar abundant, with formation of yellow pigment. Dextrose broth slightly

acid, lactose broth generally neutral. Gelatin frequently liquefied. Nitrates may or may not be reduced.

They include as valid species *M. flavus*, *M. citreus*, *M. luteus*, and *M. candicans*. It will be noted that *M. luteus* was previously mentioned as a suitable type species.

It would appear that the Winslows have supplied us with a generic diagnosis of *Micrococcus* which is usable and accurate. The genus is evidently valid, but should be written "*Micrococcus* Cohn"; not "*Micrococcus* (Hallier, Cohn) Winslow and Rogers."

Sarcina (Goodsir) Winslow and Rogers.—This genus was created in 1842 by Goodsir to include his species *Sarcina ventriculi*, discovered in the course of a microscopic examination of vomit. His work created a good deal of interest, and many papers were published during the next quarter century upon this sarcinosis. The organism was apparently found repeatedly. It is of peculiar interest because it is the first organism now included with the bacteria to be described definitely as a plant. The genus has been included in most schemes of bacterial classification down to the present time.

The species *Sarcina ventriculi* Goodsir was first cultivated by Falkenheim (1886). This investigator made use of the newly developed gelatin plate method to secure pure cultures. He found that this species would grow readily upon artificial media, forming light yellow, round colonies in from 36 to 48 hours. Packets characteristic of *Sarcina* were missing in all media tried, except hay infusion, where they developed abundantly. It would seem that this first described species of *Sarcina* might well be taken as the type of the genus.

Winslow and Rogers (1905) have proposed a somewhat radical emendation of the genus. In its later form it reads:

Facultative parasites or saprophytes. Division occurs under favorable conditions in three planes, producing regular packets. Generally decolorize by Gram. Growth on agar abundant, with formation of yellow pigment. Dextrose broth slightly acid, lactose broth generally neutral. Gelatin frequently liquefied. Nitrates may or may not be reduced.

They recognize three species, *Sarcina lutea*, *S. citrea*, and *S. flava*. To these Kligler (1913) has added *S. aurantiaca*, emending therefore the generic diagnosis by including orange as well as yellow forms.

The Winslows in a discussion of *Sarcina ventriculi* state (p. 236):

Another interesting *Sarcina*, possibly related to *S. lutea*, is the form originally isolated by Goodsir in 1842 and named by him *S. ventriculi*. It was described as a non-liquefying sarcina, isolated from the stomach in cases of hyperacidity

of the gastric juice. More recent investigations suggest that there is nothing specific in the relation of this organism to the pathological condition in question (Flügge 1896). *S. ventriculi* was distinguished from the type of *S. lutea* by the production of an orange, instead of a yellow pigment. It corresponds therefore to the non-liquefying *S. aurantiaca*. . . . Whether Goodsir's form was a packet-forming *Aurococcus* or an orange *Sarcina* can only be decided from a study of similar forms which the future may bring to notice.

The writer has been unable to find the authority upon which these authors base their statement that *Sarcina ventriculi* produces an orange pigment. Falkensheim (1885), Flügge (1886), and Eisenberg (1891) all state that this organism produces a yellow pigment on culture media. It would appear therefore that the name *Sarcina ventriculi* should have been given to one of their species, and that it should constitute the generic type. It is probable that the genus *Sarcina* Goodsir em. Winslow and Rogers is valid.

Rhodococcus Winslow and Rogers.—This generic name has been introduced 3 times into bacteriologic nomenclature. It was first used by Zopf (1891) to apply to 2 species of red bacteria the physiologic characters of which had previously been studied at length by Overbeck (1891). These organisms had been known as *Micrococcus erythromyxa* and *M. rhodochrous*. Zopf characterized the genus (or subgenus as he terms it) as follows:

Colonien auf gewöhnlicher Nährgelatine gebirgsrückenartig; roth gefärbte zellen, weder ausgesprochen fädige noch flächenförmige oder körperliche Verbände bildend, sondern unregelmässig zusammengelagert, ohne Gallerthülle, einen rothen Fettfarbstoff enthaltend, der nach der Ausscheidung in rothen, auffälligen Aggregaten krystallisirt, und durch ein einziges breites Absorptionsband bei F. ausgezeichnet ist.

These species are disposed of in various ways by subsequent authors. Migula (1900) uses the designation *Bacterium erythromyxa* (Zopf) Mig. Matzschita (1902) changed the name to *Bacillus erythromyxa*, because of its shape. He (p. 41) however uses the name *Micrococcus rhodochrous*, describing the latter as "Grosse Zellen, leine, mattglänzende, kreisrunde gewölbte, dunkelkarmin rosa gefärbte Kolonien. Auf Agar erst karmin rosa, dann tief zinnberrote Auflagerung. In Bouillon bildet sich eine dicke rosa Haut, glatt, feucht. und ein roter flockig—bröckeliger Bodensatz." This description indicates that at least one of the species is a true coccus. It would seem evident, therefore, that if the red-pigmented cocci are worthy of generic designation, *Rhodococcus* Zopf is valid.

Winslow and Rogers (1906), evidently without knowledge of the previous use of the name by Zopf, proposed *Rhodococcus* as a generic

designation for the red cocci. In their later publication (1908) they give the following diagnosis:

Saprophytes. Cells in groups or regular packets. Generally decolorize by Gram. Growth on agar abundant, with formation of a red pigment. Dextrose broth slightly acid, lactose broth neutral. Gelatin rarely liquefied. Nitrates generally reduced to nitrites, but not to ammonia.

They recognize two species only, *Rhodococcus roseus* (Flügge) Winslow and *Rh. fulvus* (Cohn) Winslow. It is apparent that this description may well be regarded simply as an emendation of that of Zopf.

Rhodococcus was independently introduced as a bacterial genus by Molisch in 1907. The only species described is named *Rhodococcus capsulatus* Molisch. This organism is described as belonging with the *Athiorhodaceae*, that group of the sulphur bacteria which contain bacterio-purpurin, but no free sulphur granules. This generic name is a homonym of *Rhodococcus* Zopf. The genus is recognized by Jensen (1909).

It would appear that *Rhodococcus* Zopf is a valid name to apply to the red cocci as a generic designation if they are to be grouped separately.

CONCLUSIONS

1. Bacteriologic nomenclature should conform as far as practicable to the International Rules for Botanical Nomenclature.

2. A study of the validity of the subfamily and generic names used by the Winslows leads to the following conclusions:

(a) Subfamily *Paracoccaceae* Winslow and Rogers. The name does not conform to Art. 23 of the code. As a substitute *Streptococceae* Trevisan em. is proposed.

(b) Genus *Diplococcus* (Weichselbaum) Winslow and Rogers. The name is invalid because of prior use of the generic name *Neisseria* Trevisan for the gonococcus. The latter designation should be substituted.

(c) Genus *Ascococcus* (Cohn) Winslow and Rogers. This probably should be replaced by the generic name *Leuconostoc* Van Tieghem.

(d) Genus *Streptococcus* (Billroth) Winslow and Rogers. The genus is valid, but the authority incorrectly given. It should be designated *Streptococcus* Rosenbach.

(e) Genus *Aurococcus* Winslow and Rogers. This name is invalid because of prior use of the name *Staphylococcus* Rosenbach, which should be substituted for it.

(f) Genus *Albococcus* Winslow and Rogers. This name is probably valid if the white staphylococci are to be accorded separate generic recognition.

(g) Subfamily *Metacoccaceae* Winslow and Rogers. The name does not conform to Art. 23 of code. As a substitute, the name *Micrococceae* Trevisan is proposed.

(h) Genus *Micrococcus* (Hallier, Cohn) Winslow and Rogers. The genus is valid, but should be designated *Micrococcus* Cohn.

(i) Genus *Sarcina* (Goodsir) Winslow and Rogers. The genus is valid, but should be designated *Sarcina* Goodsir.

(j) Genus *Rhodococcus* Winslow and Rogers. The genus is valid if the red cocci are to be accorded generic rank, but should be designated *Rhodococcus* Zopf.

SUMMARY

The Winslows' classification of the Coccaceae with the preceding corrections becomes:

A. Tribe *Streptococceae* Trevisan.

Genus 1. *Neisseria* Trevisan.

Genus 2. *Leuconostoc* Van Tieghem.

Genus 3. *Streptococcus* Rosenbach.

Genus 4. *Staphylococcus* Rosenbach.

Genus 5. *Albococcus* Winslow and Rogers.

B. Tribe *Micrococceae* Trevisan.

Genus 6. *Micrococcus* Cohn.

Genus 7. *Sarcina* Goodsir.

Genus 8. *Rhodococcus* Zopf.

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THE EFFECT OF ANAPHYLACTIC SHOCK ON THE CELLULAR REACTION OF THE PERITONEUM OF THE GUINEA-PIG *

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The work here reported had to do with the investigation of changes in the cell content of the peritoneum of the guinea-pig (as shown by serial differential counts of the peritoneal fluid made at varying intervals after the intraperitoneal injection of horse serum into guinea-pigs sensitized to horse serum), and of the reaction of the cells to determine whether this reaction differed from that obtained in the normal animal after the injection of horse serum or other non-protein substances.

Studies have been made of the cellular reaction after various injections of foreign protein into the skin. Thus, the von Pirquet reaction to tuberculin in man is known to consist largely of lymphoid cells, while the intradermal introduction of 1 c.c. of horse serum in sensitized rabbits, produces largely a polymorphonuclear reaction, according to Knox, Moss, and Brown.¹ It was thought that by studying the peritoneal fluid of the sensitized guinea-pig, the reaction to intraperitoneal injections might be found in the cells free in this cavity and the progress of the reaction in that case closely followed.

The cellular reaction, both local and general, to injections of various foreign protein substances in animals has been studied by numerous investigators. Most observers, however, have confined their attention to a study of the eosinophilia, either local or general, which may appear under these circumstances.

Schlecht and Schwenker² have noticed local collections of eosinophiles in sensitized animals at the site of re-inoculation of foreign protein. They find these in Arthus' phenomenon, in the peribronchial tissue after an intratracheal spray of the homologous protein, and they find them in the "enteritis anaphylactica" of the dog. They have noted, in from 6 to 10 days after intraperitoneal injection of foreign serum in normal animals, a marked local increase of eosinophiles, which is preceded by an increase of the pseudo-eosinophiles. After the second injection of foreign serum, however, the eosinophiles appear much more promptly.

Before studying the changes in the peritoneal fluid after any injection, it is necessary to know the normal relation of one cell type to another in the peritoneal fluid of the guinea-pig. The literature on this is scanty, dealing almost exclusively with the percentage of the eosinophiles.

* Received for publication July 24, 1915. This work was done on the Coolidge Fellowship.

1. Jour. Exper. Med., 1910, 12, p. 562.

2. Deutsch. Arch. f. klin. Med., 1912, 108, p. 405. Deutsch. Arch. f. inn. Med., 1910, 98, p. 327. Arch. f. exper. Path. u. Pharmacol., 1912, 67, p. 137.

Weinberg and Seguin³ examined the peritoneal fluid in 150 guinea-pigs. They found eosinophiles in all but 7; 18 of the 150 had over 30% of eosinophiles, and in these animals there was an increase of the eosinophiles of the blood. Stäubli, says that normal guinea-pigs have 25-31% of eosinophiles in their peritoneal fluid. Szecsi and Ewald⁵ examined the peritoneal exudate of 14 normal guinea-pigs and found the percentage of eosinophiles varying from 3 to 60%.

The peritoneal cavity of normal guinea-pigs always contains sufficient fluid for aspiration with a Wright capillary pipet. This fluid contains normally at least 4 types of cells, namely: polymorphonuclear-neutrophiles, eosinophiles, large mononuclear cells, and small mononuclear cells.

As regards the origin of these cells, the eosinophiles and polymorphonuclears doubtless come from the blood directly, being attracted by eosinotactic substances or by local irritation. Szecsi and Ewald⁵ could demonstrate parasites in all cases of peritoneal eosinophilia. But about the mononuclears, large and small, there is some discussion. Before Goldmann's⁶ work with vital staining and before the study of animals rendered aleukocytic by means of thorium according to the method of Lippmann and Plesche,⁷ there was some doubt about the origin of these two types. Some investigators, notably Weidenreich and Marchand, believed that the large and the small mononuclears were different stages in the development of the same cell, which was originally a local tissue cell. Others, headed by Maximow, thought that since the small mononuclears were morphologically identical with the lymphocytes of the blood, they came from the blood. Others, again, notably Weidenreich, Pappenheim, and Szecsi, divided all these mononuclears into hematogenic lymphatic cells and histiogenic myeloid varieties, and they believed that the epithelium of the serosa was similar in function to the endothelium of the blood vessels and could form new cells and could liberate free "Wanderzellen," which were ordinarily at rest.

Vital stains have demonstrated that both the large and the small mononuclears in the peritoneal exudate are colored, whereas pure lymphocytes, such as are found in the blood, do not take the stain. This seems to prove that both these forms are of histiogenic origin, thus confirming the original unitarian idea of Weidenreich and Marchand.

In the Lippmann-Plesch experiments with animals treated with thorium, the blood of which contained not a single leukocyte, Pappenheim⁸ obtained peritoneal exudates filled with lymphoid cells containing carmin. Hence, it is perhaps reasonable to assume that these "small lymphocytes" are not true lymphocytes, but are in fact local autochthonous cells, the so-called histiocytes. Since the large mononuclear cells occur in pericardiac exudates, and since they are different morphologically from any cell, either in the lining membrane of the pericardium or in the blood, it is reasonable to assume at least that they arise neither from the cells lining the serous cavities nor from the cells of the blood itself; their origin can therefore be spoken of simply as "histiogenic."

Throughout the present work the four types of cells have been designated by these titles: (1) polymorphonuclears; (2) eosinophiles; (3) phagocytes (large mononuclears), and (4) lymphocytes (small mononuclears).

3. Ann. de l'Inst. Pasteur, 1914, 28, p. 470.

4. Trichinosis, 1909, p. 214.

5. Folia haematol., Arch., 1913, 17, p. 167; 1912, 13, p. 1.

6. Laupp, 1912.

7. Deutsch. med. Wehnschr., 1913, 39, p. 1395.

8. Centralbl. f. allg. Path., 1913, 24, p. 997. Folia haematol., Arch., 1913, 17, p. 257.

As a starting point and foundation for further study, differential counts were made of the cells in the peritoneal cavities of 22 normal guinea-pigs of various weights. It is interesting to compare these counts (Table 1). It will be seen that the proportions of different cells varied considerably in different guinea-pigs, and that no fixed normal can be obtained.

TABLE 1
DIFFERENTIAL COUNTS OF THE CELLS IN THE PERITONEAL FLUID OF 22 NORMAL GUINEA-PIGS

Weight in Grams	Percentage of			
	Lymphocytes	Phagocytes	Eosinophiles	Polymorphonuclears
593	44.2	40.4	12.8	2.5
470	12	41	45	2
284	15	57.5	27.5	0
325	23.4	70.2	4.4	2
325	15.5	76	8.5	0
393	52.4	47.6	0	0
684	43.6	56.4	0	0
263	37.4	43	19.6	0
464	6.2	80.8	13	0
515	25.2	63.3	11.5	0
465	7.8	71	21.2	0
323	4.6	79.4	16	0
293	63.6	36.4	0	0
339	24.8	67.6	7.6	0
408	7.6	72	20.4	0
440	7.8	68	24.2	0
280	4	50.2	45	0.8
333	34.8	50.8	14.4	0
357	5.5	73	21.5	0
185	46.1	50.8	3.1	0
235	19.3	73.7	7	0
222	65	33.7	1.3	0
Average	25.71	59.22	14.73	0.34

Polymorphonuclear neutrophils were absent in all but 4 pigs, and the highest percentage was 2.6. Eosinophiles were absent in 3 pigs. They formed 45% of the cells in 2 pigs, while the next highest count gave 27.5%. The average for the 22 pigs was 14.173% eosinophiles. In only 3 pigs were both polymorphonuclear neutrophils and eosinophiles absent. The percentage of the small mononuclears and the lymphocytes varied widely, from 65% to 4%. In 4 pigs the proportion was 45% and in 7 it was below 10%. The average for 22 pigs was 25.7% lymphocytes. The proportion of the large mononuclears or phagocytes varied from 80.8% down to 33.7% and averaged 59.2%. If we add the mononuclears, large and small together, we have a much smaller variation, from 100% down to 75%, with only 3 pigs below this. The count in these gave 72.5, 54.2, and 53%, respec-

tively, and the average now becomes 84.9%. Thus most of the cells in the peritoneal cavity of guinea-pigs are of the mononuclear variety.

To demonstrate further these variations in individual guinea-pigs, a normal animal, weighing 684 grams, gave practically the same count as one weighing 185 grams, both with high lymphocytes (43.6% and 46.1%, respectively), while a guinea-pig of 465 grams gave practically the same count as one weighing 323, the proportions of the lymphocytes here being 7.8% and 4.6%. But on the whole, guinea-pigs of the same weight tend to have similar counts.

An interesting observation is that the lower lymphocyte counts tend to occur in the larger guinea-pigs; thus, of 8 pigs weighing over 400 grams, 4 had less than 8% of lymphocytes, while of 14 pigs under 400 grams, only 3 had less than 15% lymphocytes.

No definite influence could be ascribed to the time of year, to the time of day, or to the individual lots of guinea-pigs, and all the guinea-pigs were kept under the same conditions in the laboratory.

Before describing the cellular changes in the peritoneal fluid after the intraperitoneal injection of horse serum into normal and into sensitized guinea-pigs, it is necessary to describe control experiments. Each of these controls was carried out with exactly the same technic in normal guinea-pigs, varying in size but averaging about the same weight as those used later for the horse serum injections. Differential counts, each of at least 500 cells, were made of the peritoneal fluid immediately before the intraperitoneal injection, and at intervals of 15 minutes, 1 hour, 3 hours, 6 hours, 9 hours, 12 hours, 24 hours, and in some cases several days afterward. (In some of the earlier controls with non-protein substances these exact time intervals were followed only approximately.)

TECHNIC

The abdomen is carefully shaved, not only to make the introduction of the glass capillary pipette easier, avoiding all hair and extraneous matter, but also to avoid the visible blood vessels, the bleeding from which ordinarily contaminates the fluid. Drops of peritoneal fluid are then mixed on a slide with about equal quantities of an aqueous solution of brilliant cresyl blue in 1:300 dilution and covered with a cover glass. In 5 to 10 minutes all the nuclei are stained and the four main types of cells considered here are readily distinguished. For convenience, these have been here designated as "polymorpho-nuclears," "eosinophiles," "lymphocytes," and "phagocytes."

Before studying any of the control injections, the peritoneum of a normal guinea-pig, weighing 325 grams, was aspirated repeatedly at the time-intervals

enumerated, in order to determine whether repeated punctures alone might cause alterations in the proportion of cells. Such, however, did not occur; differential counts made of samples of these aspirated fluid showed approximately the same proportion of cells in all counts.

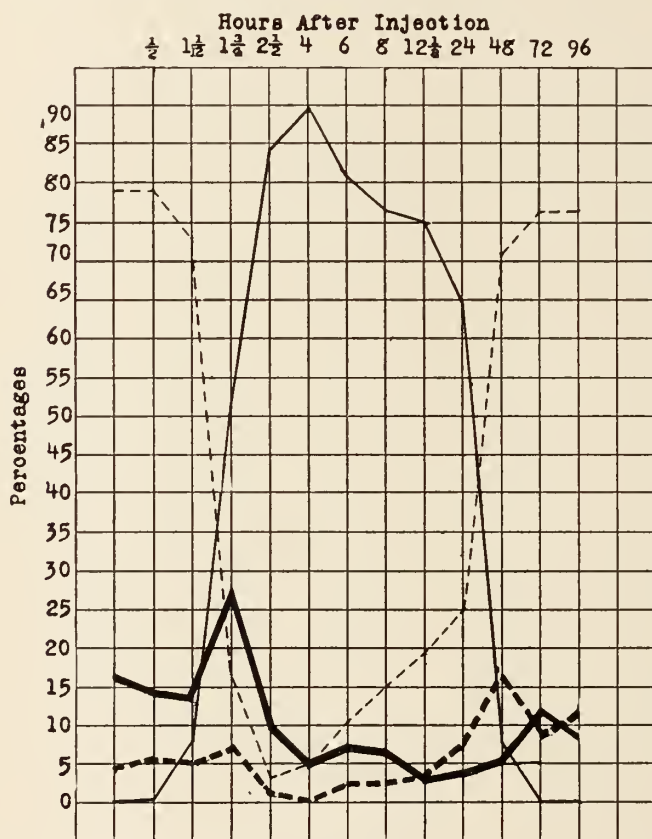


Chart 1.—The changes in the cellular content of the peritoneum of the normal guinea-pig following the intraperitoneal injection of 2 c.c. of 0.85% salt solution. The percentages refer to cells.

KEY TO CHARTS 1, 2, and 3

- = Polymorphonuclears.
- = Eosinophiles
- = Phagocytes.
- · - · - = Lymphocytes.
- = Lymphocytes and phagocytes.

Test substances as controls were injected intraperitoneally into normal guinea-pigs as follows:

(a) Two pigs, weighing 325 and 393 grams, respectively, received 1 c.c. salt solution each.

(b) Five pigs, varying in weight between 684 and 323 grams, received 2 c.c. salt solution each.

(c) One pig of 470 grams received 2 c.c. distilled water.

(d) One pig of 344 grams received 1 mg. of histamin suspended in 1 c.c. of salt solution.

(e) One pig of 293 grams received 0.1 c.c. of turpentine.

To summarize the results of these preliminary experiments, it may be said that the intraperitoneal injection of these different fluids causes in each case the same general changes in the cell content of the peritoneal fluid of guinea-pigs; the differences in the reactions to each test substance are chiefly of degree; practically all cause a polymorphonuclear rise within 4 hours, and in practically all the reaction is largely over at the end of 24 hours, since at this time the proportions of the different cells tend always to return to the individual normal. Furthermore, after this time daily counts become almost exactly alike and remain the same, even if made perhaps 10 or 20 days after the injections.

As a type of the reaction to these different substances studied, the reaction to the intraperitoneal injection of 2 c.c. salt solution may be described; and to demonstrate the different reactions to the same test substance in different guinea-pigs, Chart 1 is appended, showing the percentage changes after the intraperitoneal injection of 2 c.c. salt solution in a normal guinea-pig.

In the first 15 minutes, the percentage of phagocytes falls perhaps 8 points and that of the eosinophiles (if any are present beforehand), together with that of lymphocytes, rises. This change continues up to 1 hour; and at this time a few polymorphonuclears may be seen, perhaps 6-10%. At the end of 3 hours, there is a striking change; the polymorphonuclear neutrophiles rise from practically nothing to 60 or even 80% with, of course, a corresponding fall in the mononuclear elements, especially marked in the phagocytes. The polymorphonuclears may stay up until the sixth or even ninth hour after injection, or they may fall away again at once.

At the end of 24 hours, the proportions of the cells tend always to return to what they were before injection, and after this time daily counts become almost exactly alike, remaining the same even if made perhaps 10 or 20 days after the injection.

The reaction to the intraperitoneal injection of distilled water is in general much like that to salt solution. Histamin produces similar

changes. Turpentine, as may be imagined, causes a high rise in the polymorphonuclears, which reach at the end of 4 hours the proportion of 63%, and remain the predominating cell for from 12 to 24 hours.

It having been determined that the reaction of the cells in the guinea-pig peritoneum was much the same with all these fluids, the following main experiment was carried out:

Ten guinea-pigs sensitized to horse serum were re-injected intraperitoneally with horse serum, the peritoneal fluid being aspirated and studied immediately before the injection and at the following intervals afterward: 15 minutes, 1 hour, 3 hours, 6 hours, 9 hours, 12 hours, 24 hours, and in some cases later. Seven of these 10 guinea-pigs were sensitized by an intraperitoneal injection of 0.1 c.c. horse serum, and 3 were sensitized by an intravenous injection of 1 c.c. of horse serum. The sensitizing doses in each of the 10 guinea-pigs were given from 15 to 63 days prior to the second injection. In the first animal 3 c.c. of horse serum were given intraperitoneally at the second injection. This caused well-marked anaphylactic symptoms to appear in 10 minutes; namely, weakness, difficult respiration, and occasional twitching until death occurred 5 hours later. Subsequently a second dose of 2 c.c., given intraperitoneally, invariably produced "severe symptoms" without death. The 3 animals sensitized intravenously had only "slight symptoms."

Each of the 10 experiments was controlled by studying simultaneously or within 24 hours a normal animal of the same weight injected in the same way with the same dose of horse serum. These controls never showed symptoms. In some cases, the total number of cells per cubic millimeter of peritoneal fluid was investigated and from this the actual numbers of each particular cell calculated.

It is interesting to compare these figures with the original percentages, but, in most cases, there is little correspondence between them. This is due to the great variation in the total quantity of the fluid present, so that any great fall in the total number of cells may be real, or it may be apparent because of dilution. Hence, in this work much more stress has been laid upon the percentages than upon the actual numbers.

Preliminary counts immediately before intraperitoneal injections were made both in the sensitized and in the normal guinea-pigs. The counts made of the cells in the peritoneal fluid of the 10 normal controls have been included among the figures given in Table 1 for normal guinea-pigs. When the preliminary counts of the peritoneal fluid of the 10 guinea-pigs sensitized to horse serum (Table 2) are compared with the normal, it is seen that they differ strikingly, in that the fluid averages 10% more eosinophile cells than the normal (Table 3). This has been observed by other workers and has been commented on especially by Weinberg and Seguin.

TABLE 2
DIFFERENTIAL COUNTS OF CELLS IN THE PERITONEUMS OF GUINEA-PIGS SENSITIZED TO HORSE SERUM

Weight in Grams	Percentage of			
	Lymphocytes	Phagocytes	Eosinophiles	Polymorphonuclears
...	13.2	47.5	38.5	0.8
300	20	40.6	39.4	0
342	28.2	46.4	25.4	0
460	57.7	39.5	2.8	0
292	5.8	74.0	20.2	0
324	4.2	70.6	25.2	0
375	54	34.8	11.2	0
214	5.2	58.4	36.4	0
209	5.2	55.8	39.0	0
237	6	82.3	11.2	0.5
Average	19.95	54.99	24.93	0.13

TABLE 3
COMPARISON OF THE AVERAGE PERCENTAGES OF CELLS IN THE PERITONEAL CAVITY OF NORMAL GUINEA-PIGS WITH THAT OF GUINEA-PIGS SENSITIZED TO HORSE SERUM

	Average Percentage of Cells in Peritoneal Cavity of	
	22 Normal Guinea-Pigs	10 Guinea-Pigs Sensitized to Horse Serum
Lymphocytes.....	25.71	19.95
Phagocytes.....	59.22	54.99
Eosinophiles.....	14.73	24.93
Polymorphonuclears.....	0.34	0.13

The intraperitoneal injection of 2 c.c. of horse serum into sensitized guinea-pigs causes a somewhat different cellular reaction from that observed after the intraperitoneal injection of the same amount of horse serum into normal guinea-pigs. The effect of the intraperitoneal injection of horse serum into normal guinea-pigs does not differ from that obtained with the other control fluids, such as salt solution, distilled water, and dilute turpentine.

Charts 2 and 3 demonstrate the changes observed in the cell content of the peritoneal fluid after injections of horse serum, both in the normal and in the sensitized guinea-pig. In both the sensitized and the normal guinea-pig, within 15 minutes after the injection of horse serum, there is a marked drop in the percentage of phagocytes with a marked rise in that of lymphocytes, while that of eosinophiles remains about the same, altho tending to rise in the sensitized animal. The fluid is increased in amount in both and has comparatively very few cells,

the total number of cells decreasing in a striking manner—this decrease being perhaps more pronounced in the sensitized guinea-pig. At the end of an hour, the abdomen in the sensitized animal is almost tense with fluid and there are still very few cells. The percentage of lymphocytes now diminishes while that of the phagocytes and of the eosinophiles increases. In the normal animal this increase in the percentage of eosinophiles is more rapid than in the sensitized one.

In 3 hours after the injection of horse serum, the peritoneal fluid of the normal guinea-pig contains great numbers of polymorphonu-

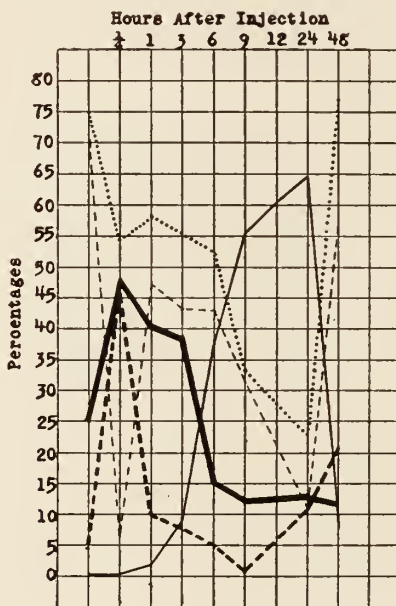


Chart 2.—The variations in the proportions of cells in the peritoneal fluid of the normal guinea-pig after an injection of 2 c.c. of normal horse serum.

clear cells, so that they form 50 to 80% of the total number; whereas in the sensitized animal at this time few of these cells are to be found and the mononuclears still predominate. In the chart shown, the polymorphonuclears formed but 9% of the cells in the peritoneum of the sensitized guinea-pig, while the mononuclears made up 53%. At the end of 6 hours the polymorphonuclears usually reached their highest percentage in the normal guinea-pig; whereas in the sensitized animal, tho they are increasing, they still form less than half the total cells. In 9 hours, the percentage of polymorphonuclears in the normal guinea-

pig may begin to decline, but in the sensitized animal it may still be slowly rising, tho even at this late date the rise is scarcely comparable to that observed in the normal guinea-pig at the end of 3 hours. Differential counts at later dates show that both in the normal and in the sensitized guinea-pig the proportions of the cells tend to return to their individual normals after from 24 to 48 hours, the polymorphonuclears disappearing, the mononuclears reappearing, tho this may be somewhat slower in the sensitized than in the normal guinea-pig. After this

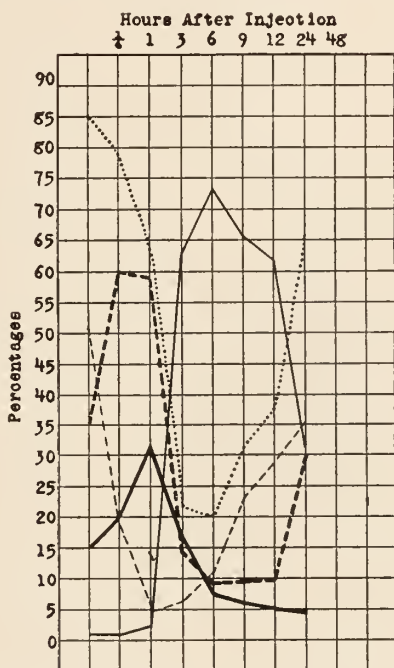


Chart 3.—Variations in the proportions of cells in the peritoneal fluid of the sensitized guinea-pig after an injection of 2 c.c. of normal horse serum.

time no definite changes can be observed in the proportions of the cells of the peritoneal fluid of either group of animals.

The changes in the total numbers of cells in the peritoneal fluid, as determined by counts made with the Thoma-Zeiss hemocytometer, have thrown no further light on the subject. In both sensitized and normal animals the total number of cells falls rapidly in 15 minutes. In one normal guinea-pig the number fell from about 11,500 to 700; in a sensitized guinea-pig, from about 27,500 to 12,000. During the

period at which the percentage of polymorphonuclears increases the total cell count increases. There is a second fall at about 9 to 12 hours during the period in which the percentage of polymorphonuclears decreases, with a final rise as the cell proportions return after from 24 to 48 hours to the individual normal.

It is evident from this description and the charts that the reaction of the peritoneum of the normal guinea-pig towards horse serum, is not essentially different from that observed towards salt solution, water, or dilute turpentine. The peritoneum of the sensitized guinea-pig, on the other hand, reacts very differently from the normal to the second injection of horse serum. Tho there are certain minor and inconstant differences, the striking and perhaps important difference following the intraperitoneal injection of horse serum is the delay in the appearance of polymorphonuclear leukocytes in the peritoneal fluid of sensitized guinea-pigs. In the normal animal these cells are present in overwhelming proportion within 3 hours after the intraperitoneal injection of horse serum, whereas in the sensitized guinea-pig they may scarcely make their appearance before this time and rarely occur in great excess before 6 to 9, or even 12, hours.

The delay in the polymorphonuclear response of the sensitized animal is of interest in the light of the experiments reported by Zinsser and Dwyer.⁹ They found that a proteotoxin, made by mixing suspensions of typhoid bacilli and normal guinea-pig serum, has the power to increase markedly the virulence of other bacteria. They found that this aggressive action of the proteotoxin is not due to the inhibition of phagocytosis; as the animals injected with it show a leukopenia, they explain the action as due to a poisoning of those tissues which ordinarily react with mobilization of the leukocytes from the circulation.

It is highly probable that a close analogy exists between the reaction of the peritoneum of the guinea-pig towards the proteotoxin of Zinsser and Dwyer⁹ and the anaphylactik shock following the intraperitoneal injection of horse serum, and it is not unlikely that any condition which interferes with the rapid outpouring of leukocytes into the peritoneal cavity will assist in the spread of infection or growth of bacteria introduced into the peritoneal cavity at this time. The reaction, therefore, of the peritoneal cavity of the sensitized guinea-pig to injections of horse serum, is very different from the reaction of the skin under the same conditions. This may be partly due to the rapid

9. Jour. Exper. Med., 1914, 20, p. 582.

absorption from the peritoneal cavity of any toxic substances that are formed. With the intracutaneous injection the absorption would be much slower.

CONCLUSIONS

There is no fixed, normal percentage relationship between the types of cells in the peritoneal fluid of normal guinea-pigs.

Old guinea-pigs tend to show a smaller proportion of small mononuclear cells than do young animals.

The peritoneal fluid of guinea-pigs sensitized to horse serum contains a higher percentage of eosinophiles than is found in the normal animal.

The reaction of the peritoneum in normal guinea-pigs to normal salt solution, water, histamin, turpentine, and horse serum, is qualitatively much the same in each case, and consists in the rapid disappearance of the mononuclear cells and the accumulation within 3 hours of polymorphonuclear leukocytes in overwhelming proportions.

The peritoneum of the guinea-pig sensitized to horse serum reacts to an injection of horse serum differently from the normal, inasmuch as the appearance of the polymorphonuclear leukocytes is much delayed.

AN OUTBREAK OF ROUP AND CHICKEN-POX IN WHICH THE HIGH MORTALITY WAS APPARENTLY CAUSED BY A SECONDARY INVADER*

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From the Agricultural Experiment Station of the University of Wisconsin, Madison, Wis.

Some outbreaks of roup and chicken-pox are attended by a high mortality; at times 85-90% of the affected birds succumb. In other outbreaks the mortality may be only 1-2%. Observations made in a number of outbreaks in which the mortality varied greatly, revealed no essential difference either as to character or extent in the lesions.

Death supposedly results from an absorption of toxins or poisons from the affected areas. This, in many instances, is perhaps the case, especially in those outbreaks attended by a low mortality, as recovery seems to be dependent largely on the extent of the lesions. (The general health of some individuals remains good in spite of extensive lesions. These cases usually recover. With this exception, the general statement holds good.) It appears hardly possible, however, that such is the case where the mortality runs high, as many birds die very suddenly before the development of extensive lesions.

An investigation by the authors of an outbreak which occurred in a large flock of poultry apparently throws some light on the subject, at least as far as this one outbreak is concerned. Following is a digest of the reports of the poultryman in charge of the plant at the time the outbreak occurred.

He purchased a number of hens which became sick shortly afterward with roup and chicken-pox. He noticed that they showed canker. A few days later other hens became sick, some showing canker, some pox scabs, and some both. Shortly after the appearance of these lesions, the hens began to die. The mortality ran at times as high as 30 to 120 per week. The poultryman was unable, as far as he could tell, to save any that showed canker. Nearly 2,000 chickens died in less than 10 months.

A bacteriologic examination of material from these lesions revealed so many different types of organisms that nothing of note was determined. However, a hen that was injected subcutaneously with a suspension obtained by macerat-

* Received for publication July 29, 1915.

ing in normal salt solution material from the canker lesions and pox scabs, became sick in 24 hours, moved very little, would not eat, developed a diarrhea on the second day, and in about 72 hours died. It was thought at this time that death was probably due to septic infection. The postmortem findings in this case were slight emaciation; catarrhal exudate in the buccal cavity and about the external nares; visceral peritoneum and oviduct slightly congested; abdominal organs otherwise normal; the right heart normal, the left dilated and filled with a semifluid dark blood clot; lungs pink in color and inflated.

Stained smears from the heart's blood, when examined under the microscope, revealed a *cocco-bacillus* 1 micron long by 0.5-1 micron wide. It would stain by the ordinary aniline dyes. Morphologically the organism resembled that of chicken-cholera. A marked leukocytosis was noted. Cultures from the heart's blood and liver on neutral agar revealed, after 24 hours, a very thin, scanty growth, translucent, and slightly beaded in appearance. Slightly heavier growth was obtained on 2% raw serum agar, a very scant growth on glycerin and lactose agar and dextrose broth, while plain broth and plain and litmus milk revealed no growth. Stained preparations from artificial media under the microscope appeared identical with those stained from the blood.

Subcultures could not be obtained except on serum agar, and then only a scanty growth. With a few exceptions, the cultures would die in from 2 to 6 days after isolation, and rarely would subcultures show any growth, even on serum agar. In order to propagate this organism, it was necessary to inject a chicken about every fourth day. Hen C1000 was injected, April 9, with a culture isolated 4 days previously, but remained well. On April 13, this same hen was injected with a culture isolated the day before. Six days later she died and the organism was recovered from the blood.

In some respects this bacillus resembles that of chicken-cholera, in that a bipolar staining is at times seen in preparations direct from the blood and it produces an acute septicemia in chickens, but differs from it markedly in its cultural characteristics in the fact that ducks are immune and injections of the killed culture will render a hen immune to subsequent injections of the living culture, which, however, will not be immune to chicken-cholera.

This organism does not cause roup or chicken-pox.—In no instance were any lesions of roup or chicken-pox produced by infection.

Injection of a killed culture confers immunity.—Hen C987, given 6 c.c. of a heated suspension, 16 days later withstood an inoculation with a virulent culture to which Hen C1000, injected at the same time, succumbed. And again, Hen A100, given 3 c.c. of the heated suspension, 7 days later withstood an inoculation of the living organism, which killed Hen A22, injected simultaneously.

Ducks are immune.—A mallard duck injected with Culture A373 remained well, while at the same time a pigeon succumbed to the

infection. Another mallard duck withstood an injection that killed Hen A316. Indian Runner Duck 983 withstood the injection that killed Hen 486.

Ducks immune to this organism succumb to fowl-cholera.—Ducks 1 and 983, as noted, withstood injections (subcutaneous) of this organism, but later succumbed to fowl-cholera.

This organism will not confer immunity to fowl-cholera.—It will be noted in the accompanying table that altho Hen C987, injected with a large quantity of killed culture, was subsequently immune to the living culture which killed Hen C989 injected at the same time, nevertheless Hen C987 when injected with fowl-cholera promptly died.

Hen	Culture Injected	Result
C987	6 c.c. of heated suspension* from infected heads	Remained well
C987	Culture A284	Remained well
C987	1 c.c. suspension of fowl-cholera from Rhode Island	Died 3 days later
C989	1.5 c.c. of unheated culture from Vermont hens	Died 4 days later. Organism isolated from heart's blood

* Injected subcutaneously.

Animals susceptible.—Besides chickens and ducks already mentioned, rabbits, mice, guinea-pigs, and pigeons were injected as follows:

Animal	Culture Injected	Days Intervening Before Death	Source of Cultures Obtained
Rabbit	A284†	1	—
Two mice	A284	(1st) 1; (2nd) 2	—
Guinea-pig	Culture isolated the day before injection	7	—
Rabbit	Culture of the day before injection	1	Heart
Pigeon	A373	2	Heart
Pigeon	Culture of the day before injection	2	Heart

† Suspension so light that no turbidity was seen.

Portals of entry.—Our experiments show that this disease is one of wound infection. It has been produced 14 times by subcutaneous injections in chickens alone as shown by the accompanying table:

Hen	Culture Injected	Days Intervening Before Death	Source of Cultures Obtained
A360	2 c.c. of light suspension of Culture C989	3	Heart and liver
A287	C989	2	Heart
A284	Culture isolated 4 days before injection	2	Heart's blood
A22	1.5 c.c. A287	Sick, but finally recovered	
C1000	Culture A284 isolated the day before injection		
A31	Culture of the day before injection	2	Heart
A373	Culture of the day before injection	2	
A254	Mixed culture	2	Heart
A316	Mixed culture	4	Heart
A49	174 and A316	1	Heart's blood
486	174 and A49	2	Heart
A99	Mixed culture	9	Heart
200	Blood tube from Hen A316	1	Heart
Ckl. C1321	Blood tube of Hen A99	6	Cultures made

An old scab was removed from the comb of Hen 174 and some virulent culture instilled. Death occurred 5 days later. A fresh wound was made on the comb and wattles of Hen A305 and some of the virulent culture instilled. This hen, however, remained well, as a result possibly of the blood's mechanically washing the organism from the wound.

The organism will not penetrate the unabraded mucous membrane when instilled into the eye and nostrils.

Hen	Seat of Inoculation	Culture	Result
A22	Eye Nostril	C989	Remained well
A246		C989	Remained well

We were unable to produce the disease by feeding either the virulent culture or fecal material from affected birds.

Hen	Culture	Result
A294	A373 A316 A49 Fed fecal matter from Hen 174	Remained well
A33		Remained well
A273		Remained well
104		Remained well

The data which we could gather as regards the biology of this organism, indicate that it probably belongs to the hemorrhagic septi-cemia group. As pointed out, under the microscope it resembles fowl-cholera but differs from it markedly in two respects:

1. Cultural characteristics. Growth was very meager, while fowl-cholera exhibits much heavier growth, will live much longer outside the animal body, and has a different appearance culturally when grown on agar slopes.

2. Pathogenicity. As already pointed out, ducks are immune to this organism, and the injection of killed cultures confers no immunity to fowl-cholera. This is a disease of wound infection, while fowl-cholera may be transmitted by way of the mouth.

Just what relation secondary invaders have to roup and chicken-pox in general throughout the country is hard to state, as unfortunately we have not had opportunity to investigate another outbreak where the mortality ran high. However, it seems reasonable to suppose that their rôle is far from inconsequential when we consider the wide range in mortality in different outbreaks in which the lesions both as to character and extent are similar.

THE TRANSMISSION AND ADMINISTRATIVE CONTROL OF MEASLES *

HAROLD FARNSWORTH GRAY

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INTRODUCTION

This paper records the experience and observations of the writer in handling an epidemic of measles in the city of Palo Alto, California, in the summer of 1914. Careful record was kept of the majority of cases, and a number of experiments in handling the outbreak were tried. In view of the evidence here presented, taken with certain recent experimental work, I propose to show that the usual methods of handling outbreaks of measles are inadequate, and I shall outline a more rational method of control.

STATISTICS OF THE POPULATION IN PALO ALTO

At the time of the epidemic, the population of Palo Alto, according to the City Engineer's estimate based on water consumption for the year, was 5,300 persons. According to the figures of the Federal Census for 1910, 51.1% of these were females. Based on a partial age distribution census, taken by the health department of Palo Alto in connection with a sanitary survey made just previous to the outbreak, the age distribution of this population expressed in percentages of the total population was approximately as follows:

Under 1 year	0.5%
2- 5	5.8%
6- 10	7.8%
11- 15	6.2%
16- 20	8.2%
21- 30	19.9%
31- 40	19.2%
41- 50	15.2%
51- 60	8.2%
61- 70	4.2%
71- 80	2.8%
81- 90	1.8%
91-100	0.2%
Total	<hr/> 100.0%

The native whites with native parents comprised approximately 56.7% of the total population; the native whites with foreign or mixed parentage approximately 23.5%; the foreign whites 16.2%; the negroes 0.5%; and the Asiatics 3.1%. With approximately 1,300 dwellings and approximately 1,370 families,

* Received for publication August 4, 1915.

the average number of persons per dwelling was approximately 4, and the average number of persons per family approximately 3.86.

A BRIEF DESCRIPTION OF THE EPIDEMIC

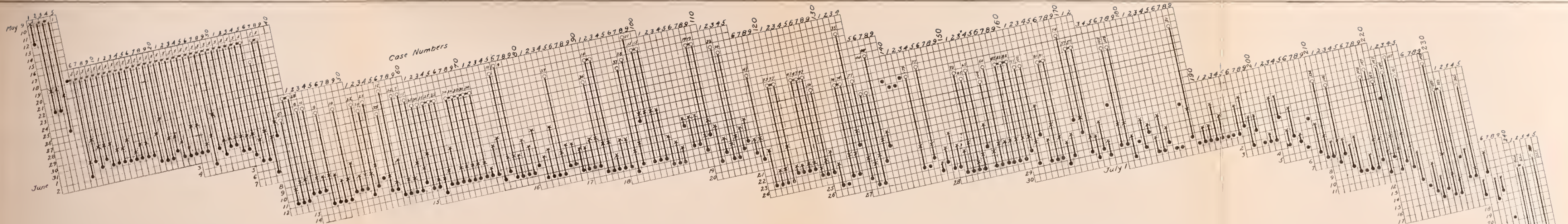
Measles had been absent from Palo Alto from June, 1913, to May, 1914; previously there had been only a few scattering cases for several years. As a result there was a comparatively large number of non-immune children, presenting a fertile soil for the development of the disease.

On May 12, 1914, a case of measles was reported in the person of C. M. G., a boy 10 years old. He had been in San Francisco sixteen days previously, but gave no history of contact with any case of measles, except that at the circus he had sat immediately in front of a girl who had a bad cold and inflamed eyes. On May 9 he felt ill; did not attend school (Saturday). On Sunday he went to Sunday school, but sat next to a boy who had previously had measles; not feeling well he did not mingle with the other children, but went directly home after the class was dismissed. He stayed home on Monday; on Tuesday he developed the typical rash. The disease was contracted by his mother and two sisters, but did not spread beyond the family.

On May 18, I. B., a boy aged 9 years, broke out with the rash of measles in one of the class-rooms in a primary school. He had apparently had a slight cold for several days, but his condition had not seemed to warrant medical attention, and his real condition had not been recognized. He had not come into contact in any way with the previous case, C. M. G., but had been to San Francisco on Sunday, May 3, fifteen days before, and as there were no other cases with which it was possible to connect him, and as it later developed that there had been many cases of measles in San Francisco at that time, it was assumed that he had obtained his infection there. The boy was at once sent home and isolated, but the entire class had been well exposed.

It was considered inadvisable to close the class-room for only one case of measles, and it was further considered advisable to keep the children in school, where they could be properly observed and sent home to be isolated on the appearance of the first suspicious symptoms. The children were examined before they entered school in the morning. The usual period of incubation being considered to be about 10 days from exposure to first symptoms, it was expected that the contacts would begin to show symptoms on Thursday, May 28, since the boy had probably not been infective on Friday, May 15, and there had been no school on the Saturday and Sunday following.

I asked Dr. H. W. Chappel to examine the children exposed to the case I. B. on the morning of the 28th, and to exclude those who were suspicious. He found 1 child with suspicious symptoms, and 2 absent. On Friday morning, May 29, I arrived at the school about 10:30 a. m. to find it practically closed, and the children I wished to examine scattered at various places in the city, practicing for the exercises to be given the following day, Decoration Day. An endeavor was made to find these children to examine them, but this was possible for only about one-half of the class, some of whom were suspicious and were ordered to be isolated. The remainder attended the Decoration Day exercises the following day, several of them, as it afterwards developed, having a coryza in the acutely infectious stage. Here they exposed



Legend

Exposure ○
 First Symptoms X
 Exanthem. ●
 Case to which exposed ○○

DIAGRAM

Showing Distribution, Date of Exposure
 Date of First Symptoms, Date of Rash,
 Period of Incubation, and Source of
 Infection in Cases of Measles in the
 1914 Epidemic in Palo Alto, California.

Harold F. Gray,
 Health Officer.
 March 31, 1915.

not only the children in their own school, but also in the three other city schools. Through a combination of circumstances the effort to confine the disease to one class-room had completely failed. The following Sunday, May 31, 9 definite cases were reported and a number of suspicious cases developed. Fifteen cases in all were reported in May.

Since practically all the school children in the city had been exposed, it was beyond the power of the department to handle the situation by morning inspection of all the children, and no funds were available to augment the department temporarily by appointing a number of physicians as medical inspectors. It was therefore decided to call the attention of the parents to the situation by closing the schools on Monday, Tuesday, and Wednesday, June 8, 9, and 10, which would be 9 to 11 days after the exposure, and sending each child home previous to the closure with a printed notice calling the attention of the parents to the situation, advising them to keep their children home during those days, watching them for suspicious symptoms, and keeping them home on Thursday also if in any way suspicious or not in normal health.

Instead of doing any good, this closure of the schools served in turn to spread the disease, as the parents in most cases let the children run wild, mingling even more than if school had been in session. The children at once started to attend the motion-picture houses, which were then immediately closed to them. If any good could have come from closing the schools, it perhaps would have resulted if the schools had been kept closed for the entire week, for the next batch of cases was reported on Saturday, June 13, and Sunday, June 14. But the conditions mentioned make it doubtful whether the closing of the schools had any beneficial effect. It probably did more harm than good.

The cases then occurred in somewhat cyclical order, with peaks about two weeks apart, comparatively few cases being reported in the intervening time. There were reported 177 cases within the city limits in June, 50 cases in July, and 1 in August, when the outbreak ceased.

At the beginning of the epidemic the department rule in regard to exclusion from school and isolation was enforced. This called for placarding, isolation, and exclusion from school of all members of the family for a period of 3 weeks from the date of appearance of the rash. The experimental work on the transmission of measles in monkeys by Anderson and Goldberger, Nicolle and Conseil, and Lucas and Prizer, showed that measles probably is not infective after convalescence is well established, and that the desquamation scales are not infective. This would indicate that a 3 weeks' isolation period is excessive, and that 2 weeks would be ample and probably 1 week sufficient. It was also believed on general principles that measles was rarely, if ever, transmitted by third persons or fomites, and that there was therefore no need to exclude from school the other children in the family during the earlier part of the incubation period. Therefore I reduced the period of isolation and exclusion from school to 10 days, and permitted contacts to attend school for

a period of 7 days after the date of probable first exposure to the infective stage of a case, which was assumed to be 4 days before the appearance of the rash. It was also decided to omit terminal disinfection as useless and a waste of funds. With a few minor variations these regulations were carried out for the greater part of the duration of the epidemic.

It should be noted in connection with the Palo Alto outbreak that the entire bay counties section was visited by an epidemic of measles during the same period, and that when all conditions are taken into consideration Palo Alto suffered relatively less in proportion to susceptible population than the other communities.

Only about one-half of the cases were seen by physicians and by them reported to the department, the remainder being diagnosed at school by the writer, or diagnosed by him at the patients' homes on report of the parents to the department. According to the best available estimate not more than 10 cases within the city limits were unreported, an estimate which would indicate a percentage of cases of measles reported exceeding 95%.

CASE RECORDS

The record of each case of measles was kept on a separate card form, and filed in a card index in the same manner as other case reports are filed. The essential information on these forms was as follows: name and age of patient, residence, school or occupation, physician, date reported, character and date of first symptoms, date of exanthem, source and date of infection, and the names, ages, and residences of persons exposed to the case; also the date when quarantined, isolated, or placarded, and the date released.

Only the cases occurring within the incorporated limits of the city were reported by the city health office to the state board of health; those within the school district, but outside the incorporated limits of the city, were reported to the county health officer, and were kept under supervision. Cases outside the city limits were recorded on the same forms as for other cases, but were kept in a separate file, and were identified by serial letters, the city cases being identified by serial numbers. In compiling the records of this epidemic for the present paper, all the cases were taken and arranged in the order in which they were reported, regardless of the date of the exanthem.

In every case the record was made from information obtained by inquiry of the parent or other person in charge of the case, and was as full as possible. Only such data were included as seemed to be reasonably exact and certain. In a few cases but little could be defi-

nitely ascertained except the date of rash; in a number of cases the data were fairly complete except for the source and date of infection, which might have been one of several cases on one or more days, in which event the source and date of infection were recorded as uncertain.

TABULATIONS BASED ON THE CASE RECORDS

In order to simplify the presentation of case records, and to exhibit the most important points graphically, I append two diagrams, the first showing 245 cases with their data on the date of exposure, the case to which exposed, the period from exposure to first symptoms, the date of the first symptoms, the period from first symptoms

TABLE 1
STATISTICS OF AN INVESTIGATION OF THE OUTBREAK OF AN EPIDEMIC OF MEASLES

From Exposure to First Symptoms		From First Symptoms to Exanthem		From Exposure to Exanthem	
Days	Number of Cases	Days	Number of Cases	Days	Number of Cases
7	1	1	21	11	4
8	4	2	62	12	23
9	15	3	73	13	34
10	37	4	40	14	34
11	24	5	17	15	16
12	25	6	3	16	9
13	15	7	4	17	3
14	4	18	1
				19	1
Average	Total	Average	Total	Average	Total
10.87	125	2.98	220	13.66	127

to exanthem, the date of exanthem, and the total period from exposure to rash; the second showing the number of cases occurring on each day of the epidemic, taken from the first diagram, and showing also the average total incubation period of the disease. These two diagrams are based on 245 of the 254 cases occurring during the same period, the remaining 9 being excluded from the table on account of incomplete data.

I next present the following tables. Table 1 shows, for 125 cases, the period from exposure to first symptoms, and gives the average period in days; for 220 cases, the period from first symptoms to exanthem, and the average period in days; and for 127 cases, the period from exposure to exanthem, and the average period in days.

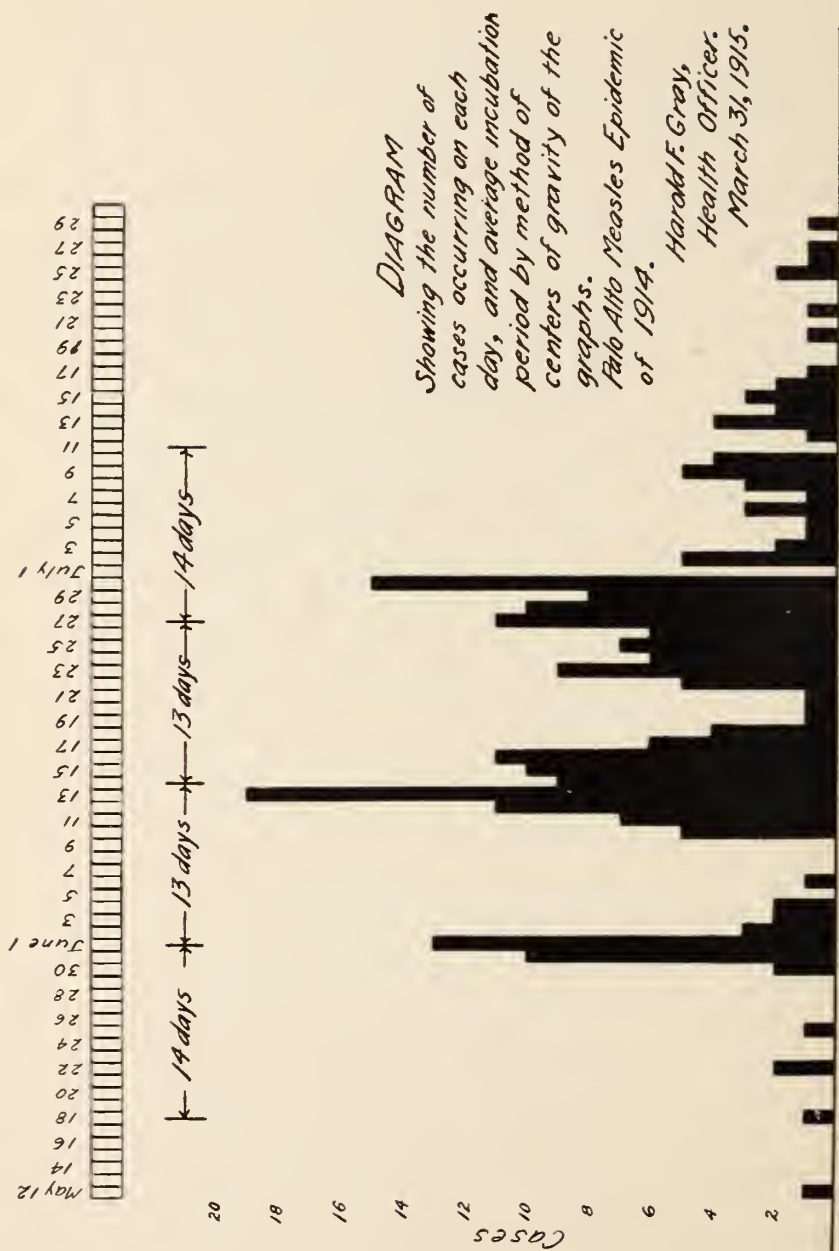


Table 2 shows, for 254 cases, the age distribution of the cases for males and females, and the totals for both sexes; also the average ages of males and females, and the average age of all patients of both sexes.

ANALYSIS OF TABULATIONS

Table 1 shows that for 125 cases for which such data were available with a reasonable degree of accuracy the minimal period in days from exposure to the appearance of the first symptoms of measles

TABLE 2
AGE AND SEX OF CASES

Age	Males	Females	Total
Under 1.....	1	...	1
1.....	6	4	10
2.....	8	3	11
3.....	6	8	14
4.....	8	7	15
5.....	8	6	14
6.....	19	8	27
7.....	15	14	29
8.....	13	23	36
9.....	13	19	32
10.....	12	3	15
11.....	6	6	12
12.....	2	2	4
13.....	4	3	7
14.....	4	5	9
15.....	1	3	4
16.....	1	1	2
18.....	2	...	2
24.....	...	1	1
28.....	...	1	1
30.....	...	2	2
31.....	1	...	1
32.....	1
33.....	...	1	1
35.....	...	1	1
40.....	...	1	1
45.....	...	1	1
Totals.....	130	124	254
Average age of males..... 7.6 years			
Average age of females..... 9.5 years			
Average age, all cases..... 8.5 years			

was 7 days, observed in one case; the maximal period was 14 days, observed in 4 cases; the average period was 10.87 days, or, for practical purposes, about 11 days.

For 220 cases for which data were available, the minimal period from the date of first symptoms to the date of exanthem was 1 day, observed in 21 cases; the maximal period was 7 days, observed in 4 cases; the average period was 2.98 days, or, for practical purposes, 3 days.

Table 1 also shows the total period of incubation in days from exposure to the exanthem, for 127 cases. The minimal period was 11 days, observed in 4 cases; the maximal period was 19 days, observed in one case; the average period was 13.66 days, or, for practical purposes, about 13.5 days. If now we turn to Chart 2, showing the number of cases reported each day during the outbreak, we find from this chart, by graphic methods, the average incubation period for the 245 cases to be 13.5 days. From the first case to the center of gravity about a vertical axis of the first group of cases we find that the incubation period was 14 days; from the center of gravity of the first group of cases to the center of gravity of the second group of cases the incubation period was 13 days; from the center of gravity of the second group of cases to the center of gravity of the third group of cases it was 13 days; and from the center of gravity of the third group of cases to the center of gravity of the fourth group of cases the incubation period was 14 days; the average of these periods was 13.5 days. This coincides very closely with the average by computation from selected cases.

Table 2 shows the distribution of 254 cases according to age and sex. Of these cases 130 were males and 124 were females—a slightly greater proportion of males, especially in view of the fact that there are slightly more females than males in the population. The average age of the females was 9.5 years, and of the males 7.6 years. The higher average age of the females was caused by two factors, one the greater number of females of mature years affected—a result largely of coming into closer contact with patients in nursing them—and also of the fact that the greatest rate of incidence among the females was at the age of 8 years, as against 6 years among the males.

The data on multiple attacks are too meager and uncertain to be of value. The data on complications and sequelae are of but little value, as a careful record was not kept of minor sequelae. The majority of cases were mild, only one serious case being reported. The only important sequelae noted were 3 pneumonias. There were no deaths.

THE STAGE OF THE DISEASE AT WHICH MEASLES IS CONTAGIOUS

During the epidemic very careful inquiry was made to determine as closely as possible the stage of the disease at which the patient infected a subsequent case. In 123 cases it was possible to trace, with comparative certainty, not only the case to which the patient had

been exposed, but the date and time of exposure. In 116 cases it was demonstrated that the patient had been in contact with one or more cases of measles, in each instance before the exanthem appeared. In a number of instances the patient had been in contact with the previous case both after and before the appearance of the exanthem; but if we assume an average incubation period of from 13 to 14 days, the time of infection must have been usually before the appearance of the rash. In 6 cases it was not possible to trace the infection at all. In no instance, however, was it possible to trace the infection to a previous case after the patient in the previous case had been released from isolation, even tho the period of isolation had been as short as 7 days from the appearance of the exanthem, and the patient released while the desquamation was still proceeding.

All the data obtained in this epidemic show very strongly that measles is contagious from the appearance of the first symptoms of the disease, but not before, and is not contagious after 7 days from the appearance of the exanthem, possibly even a less time. The height of the contagious period is probably on the day of the appearance of the rash, 73 of the 123 cases which were traced with a reasonable degree of certainty being infected at that time. In one case it seemed most probable that the infection had occurred on the second day after the appearance of the rash in the previous case. This was the only case in which the infection could have occurred after the appearance of the rash in the previous case.

In work on monkeys, Anderson and Goldberger have shown that the virus is contained in the blood and in the buccal and nasal secretions, and that 36 hours after the appearance of the exanthem the blood largely loses its infectivity. They also indicated strongly that the buccal and nasal secretions lose their infectivity with the beginning of convalescence. They completely failed to transmit the disease by means of the "scales." Nicolle and Conseil infected the bonnet monkey with blood drawn from a case of measles 24 hours before the appearance of the exanthem. Further experimental proof, corroborating these experiments in whole or in part, have been reported by Hektoen and Eggers, and by Lucas and Prizer.

This experimental evidence, taken with the epidemiologic evidence presented in the cases studied in this epidemic, seems to warrant the following conclusions:

1. A case of measles may be in the infective stage as early as 5 days before the appearance of the exanthem, but not before the appearance of prodromal symptoms.

2. The height of the infectivity occurs with the appearance of the exanthem.

3. The infectivity of the disease does not extend beyond 7 days after the appearance of the exanthem, and probably does not extend beyond the establishment of convalescence in normal cases.

PAST REGULATIONS FOR THE CONTROL OF MEASLES

Whenever there has been a lack of precise knowledge of the causative organism of a communicable disease, or of the period of infectivity and method of transmission of that disease, it has been the general principle of health authorities to make regulations which are doubtless in excess of the actual requirements. We do not know the causative organism of measles at the present time, and it is only recently that we have had any definite information as to the manner of its spread. Therefore, the restrictions placed about cases of measles have in the past been justly made in excess of the probable requirements, and even very recent regulations have been made which are excessive.

Bulletin 62 of the United States Public Health and Marine Hospital Service gives the following data on isolation periods for measles in different states:

(a) Twenty-seven days in North Carolina (may be reduced by local health authorities). Exposed persons 14 days from date of exposure.

(b) Twenty-one days in Missouri, Pennsylvania (or until physician certifies in writing that the patient is well and that nasal irritation and ear discharges have ceased), and Vermont (or until all complications are over).

(c) Fourteen days at least, or until desquamation has ceased, in Indiana, New Hampshire, North Dakota, Oregon, Utah.

(d) Fourteen days from disappearance of rash in Maryland.

(c) Ten days after appearance of last case in the house in Florida and Minnesota.

(f) Ten days in Ohio.

(g) Until recovery in Tennessee, and District of Columbia.

(h) Until desquamation is complete in Montana.

(i) Until skin and mucous surfaces are clear in New York (usually 21 days).

(j) Ten days after desquamation is complete in Idaho.

(k) Until catarrhal symptoms have disappeared in Arkansas.

Measles is required to be placed under absolute quarantine (all members of the family restricted) in Oklahoma and Montana (except breadwinners under certain precautions). The patient and exposed persons are required to be isolated in Arkansas, Kentucky, Arizona (except breadwinners), and Florida (except breadwinners). Patients and susceptible children are required to be isolated in Minnesota. The patient only is required to be isolated in the District of Columbia, Indiana, Michigan, North Carolina, North Dakota, Oregon, Utah, Vermont, and Washington. Placarding and reporting only is required in Kansas, Nebraska, South Dakota, Virginia, and Wisconsin. Measles is required by law to be reported in the District of Columbia, Alaska, Hawaii, Arizona, California, Idaho, Indiana, Maine, Maryland, Massachusetts, Montana,

New Hampshire, North Carolina, Pennsylvania, Texas, Utah, Wisconsin, and by regulation of the State Board of Health in Arkansas, Connecticut, Florida, Kansas, Kentucky, Louisiana, Michigan, Minnesota, Mississippi, Missouri, Nebraska, New York, North Dakota, Ohio, Oklahoma, Oregon, South Carolina, South Dakota, Tennessee, Vermont, Virginia, Washington, and Wyoming. No data are readily available as to the requirements in terminal disinfection in measles, but it has been rather generally practiced in the past.

The period of exclusion from school is generally the same as the period of quarantine or isolation in most states, with the following specific regulations:

- (a) Five days after release from quarantine in Indiana, Oregon, and Utah.
- (b) Fourteen days from disinfection of premises in the District of Columbia.
- (c) Fourteen days from beginning of last case in Maryland.
- (d) Twenty-one days from onset of the disease in Idaho, Montana, and

Utah.

Readmission to school is usually granted only upon presentation of a permit from the local health authority, but in 8 states may be granted upon presentation of a certificate from either the local health authority or the attending physician, or from the attending physician alone in 7 states.

No attempt has been made to digest the requirements of the different cities or other sanitary units, as the state requirements show approximately as wide a variation as the municipal requirements would show. The only municipal regulations which will be here presented are those of New York City, for the reason that they mark the most radical step yet taken in reducing the period of isolation of cases of measles. The following is quoted from the New York City Hand Book of the Bureau of Infectious Diseases for 1914, Sec. 80:

Incubation period 12-14 days. Quarantine period five days after the appearance of eruption if no catarrhal discharges are present, cough has stopped, and patient is otherwise well. On August 15, 1913, the quarantine period for measles was reduced to five days after the appearance of the eruption. That this procedure was justified is evidenced by the fact that there has been no increase in the occurrence of secondary cases.

In Sec. 53 instructions are given regarding placarding and exclusion of all children in family from school. In Sec. 55 instructions are given regarding cleansing and airing of the sick-room on termination of quarantine. On termination of quarantine school certificates are issued to the patient and to other children who have had measles; otherwise, the children are excluded for 14 days.

PALO ALTO REGULATIONS FOR THE CONTROL OF MEASLES

Previous to the epidemic the local regulations regarding measles had been as follows: The patients and all children and members of the family were excluded from school, and the premises placarded

and quarantined (modified quarantine, the breadwinner not being restricted) for a period of 3 weeks from the appearance of the rash in the last case.

The present regulations for the control of measles (adopted as regulations of the Board of Public Safety) are as follows:

General Regulations.—"Patients shall be isolated upon the premises for a minimum period of seven (7) days from the appearance of the rash, except that in cases where there are children in the same family or building who have not previously had the disease, the minimum isolation period shall be ten (10) days, or longer if necessary in the judgment of the health officer. Adults, and children who have previously had the disease, and who are not in contact with the patient, shall not be restricted. Children in the same family with a case of measles, who have not previously had the disease, and other susceptible children who have been in immediate contact with the patient, need not be isolated for a period of eight days from the time of exposure, provided they are isolated from the patient, but shall thereafter be isolated for a minimum period of ten (10) days, or until all possibility of contracting measles has passed. A warning placard, containing the word "MEASLES" in plain and conspicuous letters, shall be conspicuously affixed to the premises upon which the patient is isolated, and shall not be removed until isolation is terminated."

School Regulations.—"No child or teacher who has been excluded from school on account of a communicable disease shall be readmitted to school until he or she has been examined by the health officer or a physician appointed by the health officer for such examination, and has obtained a written permit from the health officer to return to school. Teachers shall exercise due diligence in observing children for the early signs of communicable diseases, and shall send from school any children exhibiting suspicious symptoms or abnormal conditions, and shall promptly report the fact of such exclusion to the health officer. In an epidemic of measles all children having coryza or inflamed eyes shall be excluded until all possibility of measles has passed. Children in the same family with a case of measles and who have not previously had measles, if the patient is isolated may be permitted to attend school for seven days from the first contact with the case, and shall thereafter be excluded for ten days. Children

who have previously had measles, if the patient is isolated, may be permitted to attend school at the discretion of the Health Officer."

It should be noted that in case there are other susceptible children in the family, the period of isolation is increased from 7 to 10 days. This is done for reasons of convenience and efficiency, for if the case were released at the end of 7 days it would be necessary to return at the end of about 10 days from the exposure to determine whether the other susceptible children were coming down with the disease. By lengthening the period to 10 days where there are susceptible contacts in the same family or on the same premises, one trip is eliminated.

THE MANAGEMENT OF MEASLES EPIDEMICS

One of the first difficulties which the administrative officer finds in the control of measles is the fact that, being highly contagious prior to the appearance of the rash, and but little if at all contagious after, it is seldom possible to get cases isolated until they have had considerable opportunity to spread the disease. The difficulty is still further increased by two common conditions: first, the parents of the patient usually think that the child has merely a bad cold, and do not isolate the child at once; second, in many cases no physician is called in, and the case may not be reported to the health department. Under average conditions probably not one-half of the cases of measles are reported; these act as foci for additional cases. Ambulant cases in the eruptive stage, and carriers, are rare, so that it is doubtful whether they play any significant part in the spread of the disease. The problem of control becomes, therefore, the problem of the early recognition and reporting of cases, and prompt isolation several days before the eruption appears.

It is impossible, even with the most highly organized system of medical inspection, to examine every school child every day of the school year before the child enters the schoolroom. It is possible, however, to train the teachers, who observe each child every day, to recognize the slightest departure from the normal. Every child departing from the normal can be at once sent to the school office, and there examined by the health officer, or a medical inspector, or school nurse, and excluded from school if any suspicious symptoms are present. This procedure can be worked out in any community having an organized health department, and will do much to limit the spread of other communicable diseases as well as measles. In

measles the general prodromal symptoms are coryza, inflamed eyes, fever, cough, and Koplik spots, tho aside from the increase in temperature these symptoms are not constant, some cases exhibiting one combination of symptoms, other cases another combination. Cotter¹ and Lorand,² among others, have reported cases in which Koplik spots were not observed prior to the appearance of the exanthem; but in every case observed by the writer or the local physicians before the appearance of the exanthem, Koplik spots were observed. The period before the appearance of the rash when Koplik spots were observed varied from as short as 1 day to as long as 7 days. Observations with a clinical thermometer were carried out only in the cases of certain high school students near the end of the epidemic (on account of final school examinations, these students, who would otherwise have been excluded, were permitted to attend school after observation each morning); of some 15 students so examined, 2 showed a slight rise in temperature simultaneously with the appearance of Koplik spots, and were excluded, later developing typical cases.

I am of the opinion that in each school the teachers, if no school nurse or medical inspector is available, can be taught to use the clinical thermometer, and to observe the palate and buccal membranes for Koplik spots. Then, in case the health officer cannot be at the school within a reasonable time (during which the suspected children should be kept by themselves away from the other children in school), the teachers can take the temperatures and examine the throats, and send home, with printed instructions to isolate, any children showing a temperature above normal, or Koplik spots. These children should be reported to the health officer, and investigated by him as soon as possible. In this way, even without organized school medical inspection, it will be possible to exclude from school at an early stage of the disease, and isolate, such children as are about to develop measles. Possibly under such a system a number of children will be sent home who are not about to develop measles, but as this is an error on the side of safety, and as such children can be returned to school if after subsequent examination they prove to be normal, the advantages of this method outweigh the disadvantages. A few cases might be missed by such unskilled examination, but the general gain would be considerable.

1. Arch. Pediat., 1900, 17, p. 918.

2. Jahrb. f. Kinderh., 1901, 3, 658.

At the present time the Palo Alto health department maintains in each local school an equipment to facilitate such examinations, which consists of two clinical thermometers, sterile wood tongue-depressors, 70% alcohol for disinfecting thermometers (bichlorid is objected to for use in schools on account of its highly poisonous properties), sterile cotton, record blanks, serum tubes and swabs for diphtheria, etc. The department is instructing the teachers, by means of lectures and demonstrations in the use of such equipment, in the recognition of the prodromal symptoms of the common communicable diseases, and informing them as to the rules and regulations of the department concerning communicable diseases and exclusion from school.

While these measures work out very well in practice in Palo Alto, and should work equally well in any small city with an organized health department, we have not lost sight of the fact that in order to attain any marked measure of success it is necessary to have the understanding and co-operation of the parents of school children, and to obtain the reporting of cases not in school. To attain the first object I have always, upon placarding or releasing cases, explained to the parents of patients just what the rules and regulations were, and the reason for them. Occasional use of the local newspaper for publicity concerning the necessity of reporting cases is advisable and helps greatly. In the case of children excluded from school on account of suspicious symptoms, a notice is sent with the child to the parents, setting forth the early symptoms of the suspected disease (or the disease to which the child has been exposed), advising the parents to observe the child closely for such symptoms, and notifying them that the disease must be reported to the health officer. If a new case of disease should appear among the children in school, this notice is sent home to the parents of all the exposed children. To reach those persons who do not have children in school, an advertisement is occasionally inserted in the local paper, notifying the people that cases of communicable disease must be reported, and stating what diseases are reportable; also stating that any person failing or refusing to report a case will be prosecuted. So far it has never been necessary to bring any case to trial, tho several warrants for arrest have been issued, and fines imposed. We endeavor to have people report cases as a civic duty, rather than hold the club of the law over their heads to compel them to report.

It is not necessary to accept measles as an inevitable disease of childhood, or to consider that it is impossible of control by proper measures and by the utilization of every available agency toward preventing its spread. If it had not been for an unusual and unfortunate combination of circumstances at the critical point, the epidemic which is reported in this paper would in all probability never have spread beyond the patients exposed to the original case I. B. It is entirely profitless and aside from the question to argue that children will have measles anyway, and that it is better for them to have it early in life and get it over with. This is a most pernicious doctrine, which could have been applied with equal force to any of the more serious contagious diseases, such as diphtheria or smallpox or typhoid, before we learned how to control them. Measles is at present a sufficiently important cause of death to warrant strenuous measures for its control.

CONCLUSIONS

From a study of an epidemic of 254 cases of measles when considered in connection with recent laboratory work on the disease, I believe that the following conclusions are justified:

The minimal period from exposure to first symptoms was 7 days; the maximal, 14 days; the average 11 days.

The minimal period from the appearance of the first symptoms to the appearance of the exanthem was one day; the maximal, 7 days; the average 3 days.

The minimal period from exposure to the appearance of the exanthem was 11 days; the maximal, 19 days; the average 13.5 days.

The distribution between the sexes was very nearly equal, with a slightly greater number of males than females.

The average age of cases was, in males, 7.6 years; in females, 9.5 years. The incidence was greatest in males at 6 years of age, in females at 8 years.

The cases in this epidemic were generally mild in character, only one severe case being reported. There were no deaths. The rate of attack per 10,000 of population was 487 (258 cases within the city limits in 1914 for a population of 5,300). Estimating an average case fatality of 4%, we might have expected about 10 deaths. That they did not occur may be attributed to the mild character of the outbreak, to a very general intelligent care of the cases, and to the excellent sanitary condition of the city.

Measles may be infective as early as 5 days prior to the appearance of the exanthem; the appearance of the rash marks probably the height of the infectiousness of the disease; the infectivity does not extend beyond 7 days after the appearance of the rash, and is probably shorter. The experience of the New York City department of health is that the disease is probably not infective 5 days after the appearance of the rash, and the experiments on monkeys would indicate that the infectiousness ceases with the approach of convalescence.

In view of the conclusions set forth in the previous paragraph, it is apparent that the usual regulations in force for the control of measles are excessive on the one hand and inadequate on the other.

For the control of measles it is essential that, in addition to a full and complete reporting and isolation of all cases, we take steps to make possible the early recognition of cases.

To make the early recognition of cases of measles possible, it is necessary that we educate parents as to the importance of the disease and its danger, and to train teachers to recognize the prodromal symptoms, or at least to recognize the slightest departure from the normal in any school child. Teachers can be instructed how to use a clinical thermometer, and to observe the throat for Koplik spots. Since small cities and towns, and rural districts, do not have available funds for the maintenance of adequate medical inspection, the co-operation of parents and teachers is especially important in such communities.

The essential points in regulations for the control of measles are: The case shall be recognized and isolated at the earliest possible moment; the premises upon which the case is isolated shall be placarded; the patient shall be isolated for at least 5 days after the appearance of the exanthem; adults and children who have previously had the disease need not be restricted, but it is advisable to warn them as to the slight possibility of second attacks, and keep them under observation; children who have not previously had measles and who are in contact with cases, need not be restricted for 7 days after contact, but should thereafter be isolated for at least 10 days and carefully observed. Disinfection after measles is useless and unnecessary.

Transmission of measles by third persons or fomites must be exceedingly rare, if it occurs at all.

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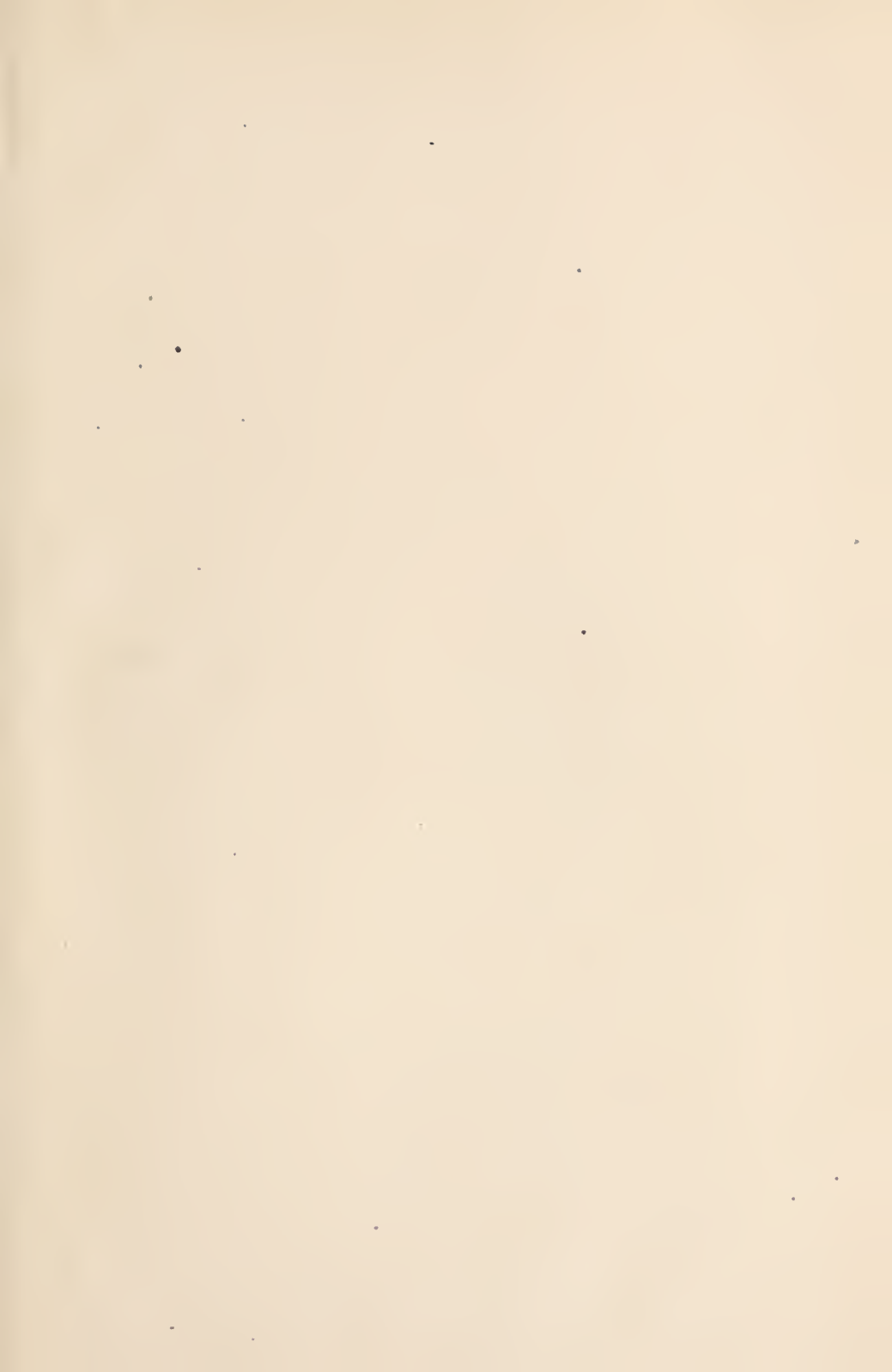
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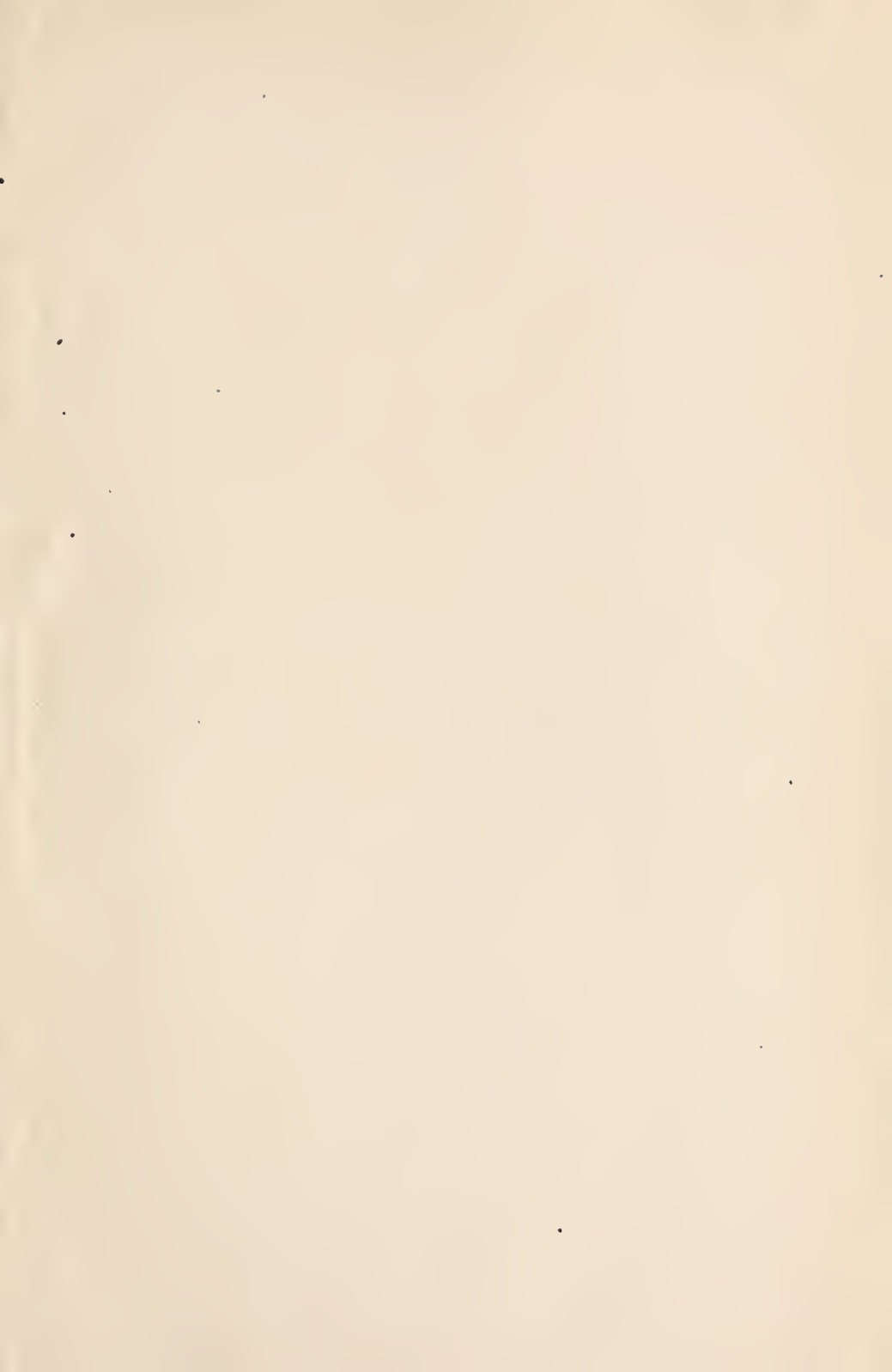
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